Cells of the haemostatic system as targets for pathogenic hantaviruses

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Mein Kind, es sind allhier die Dinge,

Gleichviel, ob große, ob geringe,

Im Wesentlichen so verpackt,

Daß man sie nicht wie Nüsse knackt.

Wilhelm Busch 1832-1908

Summary

Hantaviruses are single stranded (ss) RNA viruses belonging to the family *Bunyaviridae*. Over the past years they attracted more and more attention as "emerging viruses", increasingly gaining relevance as human pathogens. Depending on the hantavirus type they cause haemorrhagic fever with renal syndrome (HFRS) or hantavirus cardiopulmonary syndrome (HCPS). Both syndromes are associated with changes in vascular permeability, acute thrombocytopenia, and defects in platelet function. The underlying mechanisms are still poorly understood. Until now hantavirus research mostly focused on direct effects of the virus on the endothelial barrier. Although hantaviruses infect endothelial cells, no viral cytopathic effect after infection was observed. Another hypothesis claims that an uncontrolled antiviral immune response causes the vascular leakage (concept of immunopathogenesis). How pathogenic hantaviruses trigger the sharp decrease in platelet count and interfere with platelet function has not been addressed at all so far.

This thesis investigates *in vitro* whether and how hantaviruses target cells of the haemostatic system. In particular megakaryocytes (MKs), which generate platelets, and platelets themselves. We show that pathogenic Hantaan virus (HTNV), in contrast to less pathogenic Tula virus (TULV) and Prospect Hill virus (PHV), infects megakaryocytic cells. Intriguingly, after induction of differentiation megakaryocytic cells switch from low-level to high-level HTNV production without reduction in cell survival or alteration in differentiation. Thus, there is no direct viral pathogenic effect on megakaryocytic cells as previously observed for endothelial cells. Furthermore, this study demonstrates that HTNV interacts with human platelets, resulting in downregulation of essential adhesion markers, which are important for platelet aggregation and signalling. Finally, the concept of hantavirus-associated immunopathogenesis is further corroborated by showing that HTNV activates lymphocytes and triggers differentiation of monocytes towards the dendritic/macrophage cell phenotype.

Unfortunately, analysis of hantavirus-induced immunopathogenesis is hampered by the fact that no suitable animal model is available so far and normal laboratory mouse strains are not susceptible to hantavirus infection. In the future, we will use

humanized mice that permit long term human megakaryopoiesis and human immune responses to test the concept of hantavirus-induced immunopathogenesis *in vivo*.

Keywords: hantaviruses, megakaryocytes, platelets, immunopathogenesis

Zusammenfassung

Hantaviren sind einzelsträngige RNA-Viren, die zur Familie der *Bunyaviridae* gehören. In den letzten Jahren haben sie immer mehr Aufmerksamkeit als “emerging viruses” auf sich gezogen, welche zunehmend Relevanz als humanpathogene Erreger gewinnen. Sie verursachen, abhängig vom Hantavirustyp, zwei verschiedene Krankheitsbilder: das hämorrhagische Fieber mit renalem Syndrom (HFRS) und das kardiopulmonale Syndrom (HCPS). Beide Syndrome sind mit Veränderungen in der vaskulären Permeabilität, akutem Abfall der Blutplättchen (Thrombozytopenie) und Koagulationsdefekten verbunden. Die zugrundeliegenden Mechanismen sind noch immer kaum verstanden. Bis jetzt fokussierte sich die Forschung auf einen direkten Effekt des Virus auf die endotheliale Barriere. Hantaviren infizieren zwar Endothelzellen, bisher aber konnte kein zytopathischer Effekt nachgewiesen werden. Eine andere Hypothese besagt, dass eine unkontrollierte Immunantwort die endotheliale Barriere zerstört und so Hämorrhagien hervorruft (Konzept der Immunopathogenese). Auf welche Weise pathogene Hantaviren die akute Thrombozytopenie und den Funktionsverlust der Blutplättchen auslösen, ist bisher noch gar nicht untersucht worden.

Diese Doktorarbeit untersucht *in vitro*, ob und auf welche Weise Hantaviren mit Zellen des hämostatischen Systems interagieren. Zu diesen gehören insbesondere Megakaryozyten, die Thrombozyten generieren und die Thrombozyten selbst. Wir zeigen, dass das pathogene Hantaan Virus (HTNV), im Gegensatz zu dem weniger pathogenen Tula Virus (TULV) und Prospect Hill Virus (PHV), megakaryozytäre Zellen infiziert. Interessanterweise erhöht sich nach Induktion der Differenzierung in den infizierten megakaryozytären Zellen die Virusrepliaktion drastisch. Das Überleben der Zellen und der Verlauf der Differenzierung wird dadurch jedoch nicht beeinflusst. Ähnlich, wie bereits früher für Endothelzellen gezeigt, haben demnach pathogene Hantaviren keinen direkten zytopathischen Effekt auf Megakaryozyten. Weiterhin verdeutlicht die vorliegende Doktorarbeit, dass HTNV mit humanen Thrombozyten interagiert. Dies resultiert in einer Herunterregulation von essentiellen Oberflächenmarkern, die wichtig für die Aggregation und das Signalling sind. Schließlich wird das Konzept der Hantavirus-assoziierten Immunopathogenese durch Ergebnisse unterstützt, die zeigen, dass HTNV Lymphozyten aktiviert und zur

Differenzierung von Monozyten in Richtung des Phänotyps von Dendritischen Zellen/Makrophagen führt.

Leider steht noch kein Tiermodell für Hantavirus-Infektionen zur Verfügung, da normale Labormäuse nicht mit Hantaviren infizierbar sind. Deshalb werden wir in Zukunft humanisierte Mäuse etablieren, welche humane Megakaryozyten und ein humanes Immunsystem generieren, um das Konzept der Hantavirus-induzierten Immunpathogenese *in vivo* zu testen.

Schlagwörter: Hantaviren, Megakaryozyten, Thrombozyten, Immunopathogenese

Abbreviations

aa	amino acid(s)
APC	antigen presenting cell
APS	ammonium persulfate
BCCV	Black Canal Creek virus
BFU	burst-forming unit
BME	basal medium with Earls's salt
BSA	bovine serum albumin
BSL	biosafety level
$CaCl_2$	calcium chloride
CD	cluster of differentiation
CDC	Centers of Disease Control and Prevention
CFU	colony-forming unit
CTL	cytotoxic T-cell
DAF	decay-accelerating factor
DC	dendritic cell
DMSO	Dimethylsulfoxid
DNA	deoxyribonucleic acid
DOBV	Dobrava virus
ds	double stranded
dUTP	2-Deoxyuridine 5'-Triphosphate
EBOV	ebola virus
ECM	extracellular matrix
EDTA	ethylene-diamine-tetra-acetic acid
ER	endoplasmic reticulum
ERGIC	endoplasmic reticulum golgi intermediate compartment
FACS	fluorescence associated cell sorting
F-actin	filamentous actin
FCS	foetal calf serum
FFU	focus forming units
FITC	fluorescein isothiocyanate

G1	glycoprotein 1
G2	glycoprotein 2
GM-CSF	granulocyte-macrophage colony-stimulating factor
GP	glycoprotein
GPI	glycosylphosphatidylinositol
h	hours
HBSS	hanks' balanced salt solution
HCl	hydrochloric acid
HCPS	hantavirus cardiopulmonary syndrome
HEK293	human embryonic kidney cells
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
HFRS	hemorrhagic fever with renal syndrome
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSC	haematopoietic stem cell
HSV1	herpes simplex virus type 1
HTNV	Hantaan virus
HUVEC	human umbilical vein endothelial cells
ICAM- 1	intercellular adhesion molecule-1
IFN	interferon
Ig	immunoglobolin
IL	interleukin
ISG	interferon stimulated gene
JAK	janus protein tyrosine kinase
L	liter
LFA-1	leukocyte function associated antigen
L-segment	large genome segment
MAC-1	macrophage-1 antigen
MAPK	mitogen-activated protein kinases
MFI	mean fluorescence intensity
min	minutes

MK	megakaryocyte
MOI	multiplicity of infection
M-segement	medium genome segment
N	nucleocapsid
NaCl	sodium chloride
NaN_3	sodium azide
NE	nephropathia epidemica
N-protein	nucleocapsid-protein
NYV	New York virus
OD	optical density
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphat buffered saline
pc	positive control
PCR	polymerase chain reaction
PE	phycoerythrin
PGE1	Prostaglandin 1
PHV	Prospect hill virus
PI	propidiumiodide
pi	post infection
PKR	protein kinase R
PMA	4-β–phorbol-12-myristate-13-acetate
prp	platelet rich plasma
PRR	pattern recognition receptors
PSI	plexin-semaphorin-integrin
PUUV	Puumala virus
RdRp	RNA dependent RNA polymerase
ReoV	Reovirus
RGD	arginine-glycine-aspartic acid
RIG-I	retinoic acid inducible gene I
RNA	ribonucleic acid

rpm	revolutions per minute
RPMI 1640	roswell park memorial institute medium
SANGV	Sangassou virus
SD	standard deviation
SDS	sodium dodecyl sulphate
SEOV	Seoul virus
SNV	Sin Nombre virus
ss	single stranded
S-segment	small genome segment
STAT	signal transducer of activation and transcription
SUMO-1	ubiquitin-like modifier-1
TNF	tumor necrosis factor
TPO	thrombopoeitin
Tris-HCl	tris-(hydroxymethyl)-aminomethan
TULV	Tula virus
TUNEL	terminal-desoxynucleotidyl-transferase-mediated dUTP nick end labeling
TYK	tyrosine kinase
U	units
vRNA	viral RNA
VSV	vesicular stomatitis virus

Contents

1 Introduction

1.1 Hantaviruses

1.1.1 Hantaviruses as emerging pathogens

A complex interplay of pathogen, host and environmental determinants defines the spread of an infection. Due to increasing globalisation some infections gain more and more significance for public health. These "emerging infections" are pushed forward by socio-economic, environmental, ecological, and genetic factors (Jones *et al.*, 2008; Klempa, 2009; Morens *et al.*, 2008).

The World Health Organisation defines emerging zoonoses as "a zoonosis that is newly recognized or newly evolved, or that has occurred previously but shows an increase in incidence or expansion in geographical, host or vector range" (WHO, 2009). Thus hantaviruses belong to zoonotic emerging viruses because of the perpetual discovery of new hantaviruses in many different parts of the world (Europe, Asia, America, Africa) and the cyclic reemergence since detection (Schonrich *et al.*, 2008). Hantaviruses are found worldwide, but for many regions knowledge about circulating hantaviruses is still limited. Diseases caused are haemorrhagic fever with renal syndrome (HFRS) or hantavirus cardiopulmonary syndrome (HCPS). In Germany hantavirus-infections belong to the notifiable diseases since 2001. From that time on every year a few hundred cases were observed until 2007 when 1687 cases of Puumala virus (PUUV) infections occurred (figure 1). PUUV causes a relatively mild form of HFRS, the nephropathia epidemica (NE). In contrast to the epidemiological situation in previous years, where most cases occurred in metropolitan areas, the 2007 outbreak was predominantly restricted to rural areas in western and southern Germany (Hofmann *et al.*, 2008). In the same year trapping and analysis of bank voles (*Myodes glareolus*) revealed a high number of PUUV-infected reservoir hosts (Kruger, 2008).

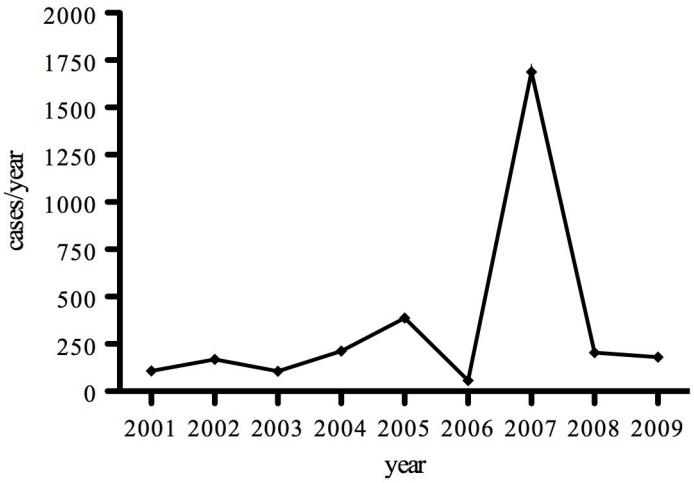

Figure 1: Diagram indicating hantavirus infections in Germany, 2001-2009.
On the y-axis cases/year, the x-axis time in years is indicated. Data from www.rki.de.

1.1.2 History

Hantavirus-associated diseases are known for hundreds of years. The first description can be found in chinese literature of the 10th century, but worldwide attention was attracted for the first time during the Korean War (1951-1953): about 3000 UN soldiers suffered from the "korean haemorrhagic fever" with clinical manifestations like fever, abdominal back pain, myalgia, chills, haemorrhages, shock and renal failure. The mortality rate was up to 15% (Smadel, 1953). The causative agent of the disease remained obscure until 1978, when Ho Wang Lee and co-workers successfully isolated the first hantavirus from lung tissue of its natural reservoir host, *Apodemus agrarius* (striped field mouse) (Lee *et al.*, 1978). Finally, in 1981, Hantaan virus (HTNV), named after the Korean river Hantaan, was propagated in cell culture (French *et al.*, 1981).

During the last years many new hantavirus strains were detected. Presently 22 hantavirus species are accepted by the International Committee of Virus Taxonomy (Nichol *et al.*, 2005). Depending on their geographical distribution hantaviruses are divided into Old World hantaviruses, circulating in Asia and Europe and New World hantaviruses, circulating in the Americas. Old World hantaviruses cause the HFRS and the milder NE, whereas New World hantaviruses are responsible for the HCPS. A selection of some important hantaviruses is listed in table 1. According to the Centers of Disease Control and Prevention (CDC) hantaviruses are potential bioweapons of

category C, because they are emerging pathogens that could be engineered for mass spread (CDC, 2009).

Table 1: Selection of some important hantaviruses circulating in Europe, Asia or the Americas and their respective reservoir host, distribution, associated disease and case fatality

Virus species	Reservoir host	Geographical distribution	Human disease	Case fatality
Hantaan virus (HTNV)	*Apodemus agrarius*	Asia	HFRS	up to 15%
Dobrava-Belgrade virus (DOBV)	*Apodemus flavicollis and other Apodemus spp.*	Europe	HFRS	1-12%
Sangassou virus (SANGV)	*Hylomyscus alleni*	West-Africa	HFRS ?	?
Puumala virus (PUUV)	*Myodes glareolus*	Europe	HFRS/NE	< 1%
Tula virus (TULV)	*Microtus arvalis and other Microtus spp.*	Europe	HFRS ?	1 case described
Prospect Hill virus (PHV)	*Microtus pennsylvanicus*	USA	-	-
Sin Nombre virus (SNV)	*Peromyscus maniculatus*	North America	HCPS	35%

HFRS: haemorrhagic fever with renal syndrome, NE: nephropathia epidemica, HCPS: Hantavirus pulmonary syndrome,

1.1.3 Structure, genome and replication

Hantaviruses belong to the family of the *Bunyaviridae*, and appear as spherical, enveloped viruses with a diameter of 78-120 nm (Goldsmith *et al.*, 1995). They have a single-stranded tripartite RNA genome of negative polarity encapsidated by the viral nucleoprotein (Obijeski *et al.*, 1976). The small (S) segment codes for the nucleoprotein, the medium (M) segment codes for the two glycoproteins G1 and G2 (also called Gn and Gc) that are cleaved cotranslationally, and the large (L) segment codes for the RNA dependent RNA polymerase (RdRp) which has further functions as replicase, transcriptase and endonuclease (Schmaljohn & Hjelle, 1997). Among hantaviruses the 3' and 5' termini of the RNA segments are highly conserved and complementary, thereby forming panhandle structures, presumably used for initiation of

transcription and replication (Hewlett *et al.*, 1977). A schematic description of a hantavirus virion is shown in figure 2.

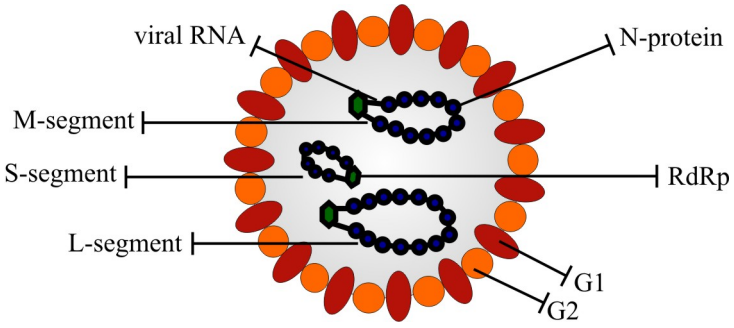

Figure 2: Schematic description of a hantavirus particle.

A lipid envelope carrying the two glycoproteins G1 and G2 surrounds three nucleocapsids consisting of distinct RNA segments; the S-segment, the M-segment and the L-segment, which form a complex with the viral N-protein and are associated with the virus-encoded RNA polymerase.

Hantaviruses enter cells via different receptors. For pathogenic hantaviruses αIIbβ3 and αvβ3, for non-pathogenic hantaviruses α5β1 were identified as receptors (Gavrilovskaya *et al.*, 1999; Gavrilovskaya *et al.*, 1998). Recently, decay-accelerating factor (DAF/CD55), a glycosylphosphatidylinositol (GPI)-anchored protein and the glycoprotein globular head domain of complement component C1q (gC1qR) were recognized to mediate infection with HTNV (Choi *et al.*, 2008; Krautkramer & Zeier, 2008).

After first contact between cellular receptors and viral glycoproteins, clathrin-dependent endocytosis takes place (Jin *et al.*, 2002). After uncoating of the virus in the cytoplasma, transcription of negative sense vRNA to mRNA is started by the RdRp. Primers for initiation of transcription are 5'-caps cleaved from cellular mRNAs (cap snatching) (Garcin *et al.*, 1995). Translation already begins when transcription is still in process. The mRNA of L- and S-segment is translated on free ribosomes in the cytoplasm (Ravkov & Compans, 2001), whereas M-segment mRNA is translated on endoplasmatic reticulum bound ribosomes. After cotranslational cleavage, glycoproteins are transported to the Golgi-apparatus for subsequent glycosylation and formation of heterodimers (Lober *et al.*, 2001; Ruusala *et al.*, 1992). Localisations of further maturation steps still have to be clarified. Most members of the family *Bunyaviridae* such as HTNV are known to bud into the Golgi-cisternae for maturation (Elliott, 1990).

However, the New World hantaviruses Sin Nombre (SNV) and Black Canal Creek (BCCV) were detected to mature at the cell surface (Ravkov *et al.*, 1997; Ravkov *et al.*, 1998). Eventually, the cell releases mature viral particles via exocytosis.

1.1.4 Reservoir hosts

Natural reservoir hosts for hantaviruses are rodents and shrews. Since hantaviruses are restricted to their natural reservoir host, a long time of co-evolution is assumed (Hughes & Friedman, 2000; Ulrich *et al.*, 2002). Nevertheless phylogenetic research showed that for individual viruses several rodent hosts and vice versa (several viruses in one host) might be possible (Muranyi *et al.*, 2005). It seems that the virus has found new ways for entering novel hosts and thereby disperses more efficiently, but although spillover can occur, geographic distribution of the virus strain is determined by habitats of the host.

Natural occurring reassortment is a rare event, but has been shown in several cases; between different Sin Nombre genetic variants (Henderson *et al.*, 1995), between different Convict Creek variants (Li *et al.*, 1995), between PUUV variants (Razzauti *et al.*, 2009), between HTNV and Seoul virus (SEOV) (Zou *et al.*, 2008) as well as between DOBV-Aa and DOBV-Af (Klempa *et al.*, 2003). Also *in vitro* reassortment was demonstrated, e.g. between Sin Nombre and Black Creek Canal virus (Rodriguez *et al.*, 1998) and between Dobrava Slo/Af and Dobrava Sk/Aa (Kirsanovs *et al.*, unpublished data).

Transmission between rodents takes place horizontally, mainly through aggressive behaviour (e.g. bites) (Botten *et al.*, 2002; Lee *et al.*, 1981). In the beginning of hantavirus research it was believed that viruses do not cause any symptoms in their reservoir host (Childs *et al.*, 1989), but since 1996 some evidence has been provided that rodents infected with New York virus (NYV) or SNV show pulmonary oedema and periportal hepatitis (Lyubsky *et al.*, 1996; Netski *et al.*, 1999). Transmission to other animal species like moose, cat or dog, can occur as spill over but no persistent infection has been described in these animals (Muranyi *et al.*, 2005).

1.2 Hantavirus-associated clinical syndromes in humans

1.2.1 Clinical course

Transmission to humans occurs via the respiratory route by inhalation of aerosols from rodent saliva, urine and faeces. Hence, the respiratory tract is the first site of infection (McCaughey & Hart, 2000). Nevertheless, transmission by bites has been observed (Douron *et al.*, 1984). Humans are dead end hosts for hantaviruses as no transmission to further hosts occurs. Solely for Andes virus person-to-person transmission has been reported (Enria *et al.*, 1996; Padula *et al.*, 1998), a controversial issue discussed among hantavirus researchers. Although hantaviruses cause no lethal symptoms in their natural hosts they can induce severe disease in humans. Case fatality rates are up to 15% (Lee, 1989; Lee *et al.*, 1978) for HFRS and 0.15% for NE (Brummer-Korvenkontio *et al.*, 1980; Mustonen *et al.*, 1998). The typical symptom observed is renal failure. Cardiopulmonary syndrome, caused by new world hantaviruses, is associated with a lethality rate of 30-50% (CDC, 2008; Nichol *et al.*, 1993). HCPS is characterized by pulmonary oedema and respiratory distress. Although three different syndromes are observed, boundaries between symptoms are flowing and some common features may be shared. In the following chapter HFRS an NE will be explained in more detail.

1.2.2 HFRS and NE

First symptoms are observed approximately 2-4 weeks after infection. The clinical course is divided into fives stages: (1) febrile phase, (2) hypotensive phase, (3) oliguric phase, (4) polyuric phase and (5) convalescent phase. The first phase of about 3-5 days is characterized by chills, myalgia, headache, vomiting and blurred vision (Schonrich *et al.*, 2008). Platelet count decreases (thrombocytopenia) with platelet count below 150.000 x 10^9 platelets/L and platelet function may be defective (Lee, 1987). Along with this decrease haemorrhages like petechiae, bleedings of mucosa, hematuria and epistaxis can occur. In addition disseminated intravascular coagulation is possible (Lee *et al.*, 1983). The hypotensive phase lasts few hours to 2 days. Typical symptoms are hypotension, shock and leukocytosis. Renal function is derogated. This can result in proteinuria, oliguria (or anuria) and abnormal urinary sediment in the following oliguric phase (2-7 days). During the following polyuric phase kidney function returns to a normal level but polyuria still creates problems with fluid and electrolyte management

(Peters *et al.*, 1999). Recovery during the convalescent phase can persist for several months. NE is a mild variant of HFRS with symptoms similar to those of HFRS but less severe (Muranyi *et al.*, 2005; Schonrich *et al.*, 2008). So far no antiviral therapy and no WHO-approved vaccine exist. Thus treatment of patients is only supportive. (Maes *et al.*, 2009).

1.3 Hantavirus-induced pathogenesis in humans: direct viral effects?

Many aspects of hantavirus-induced pathogenesis are unclear. Direct and indirect viral effects are discussed. Hantaviruses infect endothelial cells, which build a selective barrier between the vessel lumen and surrounding tissue, but so far virus-induced cytopathic effects were not detected in endothelial cell cultures although hantavirus-induced cell death of some cell lines has been reported. Moreover, it has been suggested that interaction of pathogenic hantaviruses and integrins could lead to disruption of important endothelial cell functions leading to haemorrhages (Gavrilovskaya *et al.*, 1999; Gavrilovskaya *et al.*, 2002; Gavrilovskaya *et al.*, 1998). Alternatively, the virus could cause symptoms indirectly by inducing a massive and uncontrolled immune response, which eliminates the virus at the cost of considerable collateral damage, a process called immunopathogenesis (Khaiboullina & St Jeor, 2002).

1.3.1 Cell death

The phenomenon of "cell death" can be subdivided into three different categories: apoptosis (type I), autophagy (type II), and necrosis (type III). Boundaries are floating and especially morphologies of type I and type II overlap (Lockshin & Zakeri, 2004).

Apoptosis is the programmed cell death, a suicide program of the cell. It is part of the cellular metabolism. This implies that the whole process is under stringent control. During apoptosis shrinkage of cells, condensation of chromatin, nuclear fragmentation and membrane blebbing is observed. Apoptosis can be driven by intrinsic (the cell itself induces apoptosis) or extrinsic (apoptosis is induced by outer stimuli) signals. Autophagy is a major pathway for the degradation of long-lived proteins and cellular organelles via lysosomal machinery. Vacuolisation, degradation, recycling of cytoplasmic contents and little chromatin condensation are observed. It is assumed that autophagy mediates apoptosis (Hsieh *et al.*, 2009; Lockshin & Zakeri, 2004). Necrosis

is a passive, degenerative cell death. It is characterized by complete disintegration of organelles and results in uncontrolled lysis of the cell and inflammatory reactions (Galluzzi *et al.*, 2007). After viral infection all kinds of cell death are possible: apoptosis/autophagy driven by the cell for clearance of the virus and necrosis as an uncontrolled inflammatory reaction due to viral replication.

Cell death after infection with hantaviruses is a controversially discussed topic. Apoptosis was detected in VeroE6 cells after infection with HTNV and PHV (Kang *et al.*, 1999). In human embryonic kidney cells (HEK293) a cytopathic effect was observed 3-4 days after infection with different pathogenic hantaviruses (Markotic *et al.*, 2003). In addition triggering of proapoptotic death was observed after infection of VeroE6 cells with Tula virus (Li *et al.*, 2005). Other studies revealed different results. Pensiero *et al.* could not show cytopathic effects after infection of endothelial cells with HTNV (Pensiero *et al.*, 1992) and no apoptosis was detected after infection of primary dendritic cells with HTNV (Raftery *et al.*, 2002).

1.4 Integrins

Integrins enclose a family of highly conserved proteins found in multifarious species, ranging from sponges to mammals. They are transmembrane receptor proteins that consist of two non-covalently bound glycoprotein subunits. As integrins are always composed of one α and one β subunit, they are called heterodimeric receptors. Currently, in mammals 19 α and 8 β subunits are known to form 25 distinct integrin-receptors (Humphries, 2000; Hynes, 1987). Many integrins harbour alternative splice variants, thus increasing their variety on protein level. For example, for β1-integrin five different isoforms are known (β_{1A}, β_{1B}, β_{1C}, β_{1C2}, β_{1D}) (Fornaro & Languino, 1997; Svineng *et al.*, 1998). Variants are expressed on different cell types and may differ in their function (Flier & Sonnenberg, 2001).

Integrins are composed of a large extracellular domain and a short cytoplasmic tail. Both are involved in ligand recognition. That implies integrins are able to intercede bidirectional signalling: “outside-in” as well as “inside-out” (Ginsberg *et al.*, 1992; Shattil *et al.*, 1998). Most integrins bind more than one ligand and most ligands bind more than one integrin (Hynes, 1992). That makes the receptors potent mediators in cell-cell interactions. In addition most integrins are not constitutively active: they exist

in three different conformations, inactive (low affinity), active (high affinity) and ligand occupied. By different conformations integrin function is regulated (Askari *et al.*, 2009).

Integrins are participants in versatile biological processes. Their primary function is mediating interaction of cells with the extracellular matrix (ECM). For adhesion integrins initiate remodelling of the cytoskeleton forming specialized structures. These structures, called focal adhesions, are bunched actin filaments connected to integrins (Hynes & Lander, 1992; Flier & Sonnenberg, 2001). Furthermore, integrins are important for cell-cell contacts, signal transduction, proliferation, apoptosis, differentiation, migration, the immune response, haemostasis and play a key role in many human diseases, e.g. Glanzmann's disease, a bleeding disorder (Giancotti & Ruoslahti, 1999; Hynes, 2002; Nurden, 2006; Schwartz *et al.*, 1995; Flier & Sonnenberg, 2001).

1.4.1 Hantaviruses and integrins

As mentioned above hantaviruses use integrins to enter the cell. Raymond *et al.* showed that HTNV only binds to the inactive form of β3 integrin and that binding to the receptor is independent of the presence of the RGD (arginine-glycine-aspartic acid) peptide motif, a general binding motif of integrins, but dependent on PSI (plexin-semaphorin-integrin) domain that is found at the apex of inactive αvβ3 (Raymond *et al.*, 2005). Moreover, it was shown that Andes virus uses the PSI domain of β3 for entry (Matthys *et al.*, 2009). No motif-dependent use of β1 by apathogenic hantaviruses has been shown so far. β1 integrin can be found on nearly all cell types. Expression of β3 is more differentiated. It is expressed together with αIIb as a receptor on platelets and megakaryocytes, together with αv it is found on a wide variety of cells such as endothelial cells, smooth muscle cells, mast cells and monocytes.

1.5 Haematopoiesis

Haematopoiesis is the formation of cellular blood components. This capacity for renewal of the haematopoietic components relies on haematopoietic stem cells (HSCs), which possess a high proliferative potential. The haematopoietic system provides a constant supply of all blood cells and very distinct functions can be attributed to at least

10 different cell types present in the peripheral blood. Among these are megakaryocytes and platelets, playing a crucial role in haemostasis. A striking, and so far unresolved, feature of hantavirus-associated pathogenesis is the acute loss of platelets, which are derived from mature megakaryocytes. The cause for hantavirus-associated thrombocytopenia can have two reasons: 1) decreased production of platelets due to defects in the process of platelet formation, the megakaryopoiesis and thrombopoiesis or 2) destruction of platelets in peripheral blood.

1.5.1 Megakaryopoiesis and thrombopoiesis

Megakaryocytes, the precursors of platelets, are, with a diameter of 40-50 μm, the biggest cells in the bone marrow. They originate from pluripotent haematopoietic stem cells, which mature and further differentiate to finally release platelets.

Megakaryopoiesis, the formation of megakaryocytes, is a multiple-stage development. Pluripotent haematopoietic stem cells, that carry the stem cell surface marker CD34, differentiate to the primitive MK progenitor cells, called burst-forming unit-MK (BFU-MK). Out of these cells the more differentiated colony forming unit MK (CFU-MK) are formed. These cells hold a high proliferation activity (Briddell *et al.*, 1989). In the next step promegakaryoblasts followed by megakaryoblasts develop. Approximately at this point in time cell division is arrested and cells undergo endoreplication. Thus cell size is enlarged, DNA content is increased and a cell with a multilobular nucleus and DNA content of 16-64n is generated (Odell *et al.*, 1976). Megakaryocytes with lower DNA content have less thrombopoietic potential since MK ploidy is connected to functional gene amplification (Odell & Jackson, 1968; Raslova *et al.*, 2003). It is assumed that CD34 expression is downregulated over time and expression of integrins αIIb (CD41), β3 (CD61) (forming receptor αIIbβ3) and CD51 is increased (Debili *et al.*, 1992). Expression of CD41 is a marker for onset of haematopoiesis, but only expression of the receptor αIIbβ3 results in commitment towards the megakaryocytic lineage (Emambokus & Frampton, 2003; Mikkola *et al.*, 2003). Subsequently, additional typical markers can be identified on the surface of the cells, e.g. CD42 (a multimer combining the glycoproteins CD42a (GPIX), CD42b (GPIbα), CD42c (GPIbβ) and CD42d (GPV)). During development cellular equipment has changed significantly and all typical megakaryocyte- and platelet-proteins have

been synthesized. The next step is the terminal maturation into proplatelet-forming megakaryocytes. These cells, juxtaposed to the blood vessels, form long cytoplasmic protrusions; designated proplatelets. The proplatelets protrude across the endothelial barrier into the blood stream, where mature platelets are released by cytoplasmic constriction at the very ends of the extensions (Italiano *et al.*, 1999; Junt *et al.*, 2007; Radley & Scurfield, 1980). Main regulator of the development processes is thrombopoietin (TPO), a glycoprotein hormone (Kaushansky, 1995). Altogether, the interaction of many cytokines, receptors and matrix proteins leads to the formation of the mature megakaryocyte and ultimately platelets. Figure 3 depicts a summary of megakaryopoiesis and thrombopoiesis.

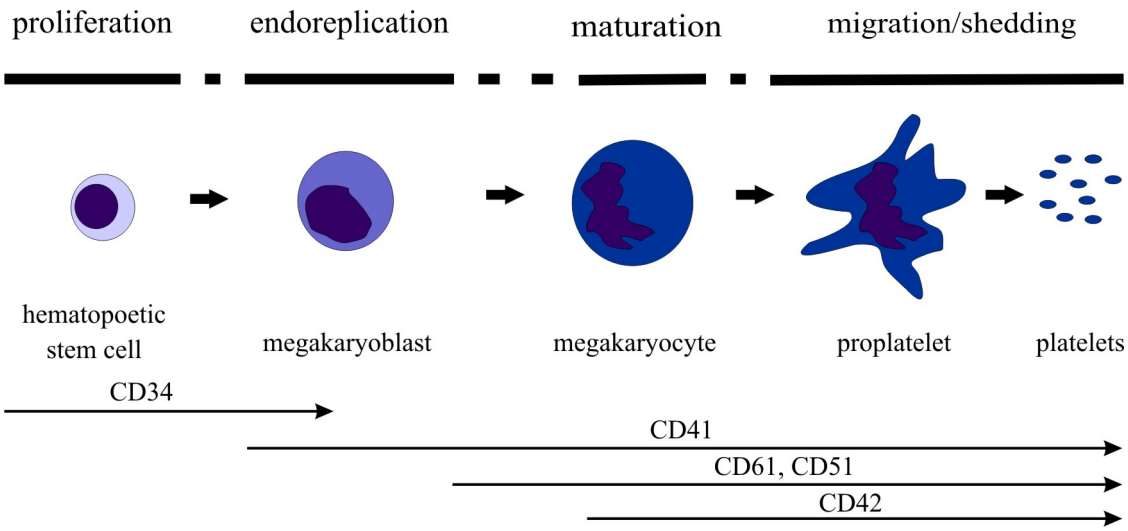

Figure 3: Scheme of megakaryopoiesis and thrombopoiesis.

Haematopoietic stem cells differentiate, via several steps of proliferation and maturation into the mature megakaryocyte. Megakaryocytes form extension of the cytoplasma, designated proplatelets. At the ends of the extensions platelets are produced by cytoplasmic constriction. During megakaryopoiesis CD34 is downregulated and megakaryocyte/platelet proteins like CD41, CD61, CD51 and CD42 are upregulated.

1.5.2 Platelets

The cellular components of coagulation are platelets. In 1882 Giulio Bizzozero discovered platelets as a component of the blood involved in coagulation after vascular injury (Bizzozero, 1882). Since this finding a great deal of research has been done in the platelet field.

Platelets are anucleate, discoid shaped cells (≈ 0.5 – 3.0 µm). Out of one single megakaryocyte approximately 1000 functional platelets can be produced. Mitochondria

as well as enzymes of glycolysis and pentose-phosphate-pathway ensure survival of platelets. After a lifespan of 7-10 days they are degraded in liver and spleen. The physiological cell count in human blood is 150-400 x 10^9/L.

Next to mitochondrial DNA, platelets contain low amounts of mRNA that is used for active protein biosynthesis (Kieffer *et al.*, 1987). Platelets possess various molecules, which function as receptors for agonists and adhesion. In addition, platelets imply three different kinds of granules: δ–granules, α-granules and λ–granules. The δ–granules, the smallest granules, contain nucleotides, amines, and bivalent cations. The α granules store platelet specific proteins (e.g. platelet factor 4) and plasma proteins (e.g. thrombospondin) that are involved in haemostasis, inflammation, healing and adhesion (Niewiarowski *et al.*, 1994). The λ–granules contain digestive enzymes, acid proteases like cathepsin D and E and glycohydrolases e.g. heparinase (Rendu & Brohard-Bohn, 2001).

Next to their fundamental role in haemostasis platelets are also important immune cells. They contain various bioactive compounds, which are released upon activation and are able to phagocytose immuncomplexes and viral particles (Loffler *et al.*, 1997).

1.5.3 Haemostasis

Haemostasis is the sum of all physiological processes that stop bleedings. It is an essential process to sustain the integrity of the vascular system after injury. Thereby loss of blood is minimized. The major participants of this process are platelets, the endothelium and the blood-plasma with its various coagulation factors.

Haemostasis is divided into two phases: primary and secondary haemostasis. During primary haemostasis vasoconstriction (the narrowing of the blood vessel), adhesion and activation of platelets takes place at the site of vascular injury. In secondary haemostasis the already formed clot is sustained by addition of fibrin (Teller & White, 2009). Integrins and their ligands, important for adhesion and aggregation, play a key role in haemostasis (Ni & Freedman, 2003). As soon as any member of the processes mentioned above is defect, haemostasis can be disturbed and haemorrhages develop.

1.6 Immune mechanisms of hantavirus-associated pathogenesis

1.6.1 The immune system

The immune response is subdivided into two parts: innate and adaptive. The innate immune response is activated immediately after infection by the pathogen in a non-specific manner. It is the first barrier for the pathogen to overcome. The innate immune response relies upon germline-encoded conserved receptors (pattern recognition receptor (PRR)) recognising conserved structural moieties on pathogens (pathogen associated molecular patterns (PAMPS)). Cells of this first barrier are monocytes/macrophages and neutrophils. These cells are able to phagocytose the invaded microorganisms and induce further defence mechanisms. Innate immunity is able to discriminate between self and non-self efficiently, but no long lasting protection is developed (Alberts *et al.*, 2002; Janeway *et al.*, 2002).

When a pathogen manages to survive the attack by the innate immune system the adaptive immune response is induced. The adaptive immune system reacts in a specific way and is characterized by adaptation of the cells to the foreign antigen. It is comprised of antigen presenting cells (APC), like dendritic cells (DC), of T-lymphocytes (responsible for the cellular response), and of B-lymphocytes (important for humoral response). Through clonal expansion highly specific immune cells are generated that eliminate the pathogen. Additionally, memory cells are produced that prevent future infections with the same microbe (Alberts *et al.*, 2002; Janeway *et al.*, 2002).

1.6.2 Hantavirus-induced immune response

The immune system reacts already four hours after hantavirus infection (Prescott *et al.*, 2005). Dependent on virus pathogenicity different amounts of interferon stimulated genes (ISGs) are upregulated. It was shown that one day after infection with non-pathogenic PHV expression of ISGs is strongly induced in endothelial cells whereas pathogenic HTNV and New York-1 virus (NY-1V) stimulate expression of ISGs not prior to four days post infection (Geimonen *et al.*, 2002). Furthermore, retinoic acid inducible gene I (RIG-I) a cytoplasmic receptor, that recognizes blunt ended or 5'-overhang ds (double stranded) RNA ends and uncapped ssRNA 5'-triphosphate ends (Yoneyama *et al.*, 2008) has been analysed in the context of hantavirus infection. Lee *et al.* (unpublished data) showed that RIG-I replication inhibits proliferation of

hantaviruses and that viral N-protein RNA stimulates RIG-I. This effect is dependent on virus pathogenicity. In contrast, the cytoplasmic tail of the G1 protein of NYV, but not of PHV blocks RIG-I directed interferon-I (IFN-I) transcription (Alff *et al.*, 2006). Activation of PRRs leads to secretion of inflammatory cytokines and expression of antiviral proteins. Thus, pathogenic hantaviruses evade type-I interferon response. Accordingly, delayed expression of MxA, an important ISG, was demonstrated after infection of primary endothelial cells with pathogenic HTNV and NYV in contrast to rather nonpathogenic TULV and PHV (Geimonen *et al.*, 2002; Kraus *et al.*, 2004).

Dendritic cells can be infected with HTNV *in vitro*. However, HTNV-infected dendritic cells do not undergo apoptosis after infection (Raftery *et al.*, 2002) in contrast to other viruses such as HSV (Muller *et al.*, 2004). Instead they fully mature and acquire T-cell stimulatory capacity (Raftery *et al.*, 2002). The amount of CD8-T-cells is increased after hantavirus infection and the CD4+/CD8+ T-cell ratio is reversed (Chen & Yang, 1990; Van Epps *et al.*, 2002). CD8-T-cells are important for clearance of infected cells, but thereby destroy endothelial cells that are important for vascular integrity. Thus, lymphocytes are discussed to mediate immunopathogenesis leading to haemorrhages. Supporting this notion, the frequency of virus-specific CD8-lymphocytes was shown to correlate with the severity of disease (Kilpatrick *et al.*, 2004).

The viral nucleoprotein is the most abundant component in humans after infection and the early humoral response is basically directed against viral N-protein (immunoglobolin A (IgA), IgM, IgG) (Vapalahti *et al.*, 2001). Antibodies directed to N-protein are detected early after infection (Kallio-Kokko *et al.*, 2000; Lundkvist *et al.*, 1993; Lundkvist *et al.*, 1996), whereas antibodies directed to viral G-proteins can only be detected later on (Maes *et al.*, 2004). Although the virus is eliminated by the human host, neutralizing antibodies can be detected many years after infection, suggesting life-long protection against reinfection (Settergren *et al.*, 1991; Ye *et al.*, 2004). So far published results provide some evidence for the concept of immunopathogenesis. However, immune responses after hantavirus infection in humans remain to be analysed in more detail.

1.6.3 The concept of immunopathogenesis

During hantavirus infection haemorrhages and decreased platelet count are striking and life-threatening symptoms, but how they are caused is still poorly understood. Bleedings are caused by disturbance of the integrity of the endothelial barrier. It is known that hantaviruses infect endothelial cells *in vivo* and *in vitro* (Zhu, 1988), but so far no direct cytopathic effect was observed. Therefore, haemorrhages probably occur due to a mechanism other than direct virus-induced damage of the cell. One feasible explanation is offered by the concept of immunopathogenesis (figure 4): an uncontrolled excessive immune response of the host (Khaiboullina & St Jeor, 2002).

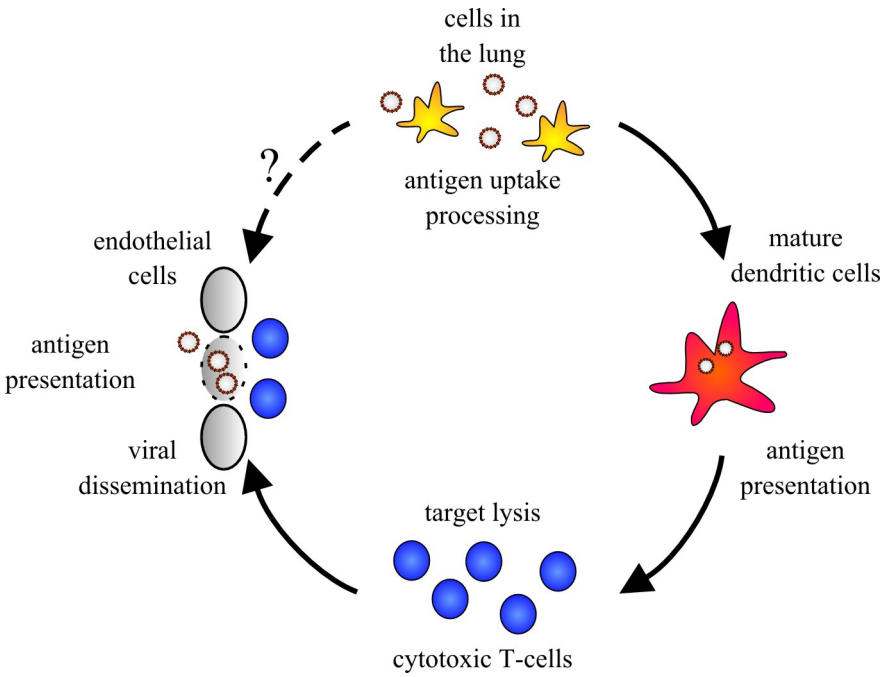

Figure 4: Working hypothesis of hantavirus-induced immunopathogenesis in humans.
Viral particles are taken up in the lung where DCs are among the first cells that are infected. They can function as carriers and transport the virus throughout the human body presenting hantaviral antigen on their surface. Furthermore, hantaviruses stimulate DC maturation. Thus T-cells can be stimulated and virus specific effector cells are generated. Endothelial cells are infected and also present hantaviral antigen on their surface. By this they are becoming potential targets for virus-specific CTLs. Hence the endothelium is disturbed leading to increased permeability of the endothelial barrier and increased viral dissemination.

1.7 Biosafety in Hantavirus research

According to Centers for Disease Control and Prevention agents with potential for aerosol transmission and disease that may have serious or lethal consequences must be handled under biosafety level 3 (BSL-3) conditions (CDC, 2007). Thus, experimental procedures with hantaviruses must be accomplished in a BSL-3 laboratory. This implies that primary and secondary barriers to protect people from exposure to potentially infectious aerosols must exist. Nevertheless, in recent years several infections with hantaviruses after laboratory work occurred (Desmyter *et al.*, 1983; Lloyd *et al.*, 1984; Tsai, 1987; Umenai *et al.*, 1979), indicating the importance of high biosafety when working with hantaviruses. For this reason experiments with hantaviruses are cumbersome as well as time-consuming and experimental possibilities are more restricted in contrast to research under biosaftey level 1 or 2.

1.8 Objectives

The hallmarks of hantavirus infection are haemorrhages and thrombocytopenia, but why these symptoms occur is not completely understood. The aim of this study is to provide a better understanding of the mechanisms underlying these symptoms. Therefore, the interaction of hantaviruses and cells of the haemostatic system, i.e. megakaryocytes and platelets, will be analysed. Moreover, the human immune response after hantavirus infection will be investigated. The questions that will be solved are: (1) Do different hantaviruses infect megakaryocytes and/or platelets? (2) Is formation of platelets abnormal, resulting in less or defective platelets? (3) Do hantaviruses directly influence megakaryocyte/platelet function? (4) How is the immune system involved, and is the thesis of hantavirus-associated immunopathogenesis supported?

2 Material and Methods

2.1 Material

2.1.1 Cells and cell lines

Cells	Description	Source
HEL cells	human erythroleukemia cell line	kindly provided by Lüder Wiebusch
Huh7	human hepatoma cell line	ATCC nr.: CCL-185
K562 cells	human chronic myeloid leukemia in blast crisis	kindly provided by Harald Schulze
L8057 cells	murine megakaryoblastic cell line	kindly provided by Harald Schulze
Meg-01 cells	human chronic myeloid leukemia in megakaryocytic blast crisis	kindly provided by Monika Bigalke
monocytes	primary human monocytes	isolated from buffy coats
PBMCs	peripheral blood mononuclear cells	isolated from buffy coats
platelets	primary human platelets	isolated from freshly drawn blood
TF-1 cells	human erythroleukemia cell line	kindly provided by Carmen Scheibenbogen
VeroE6 cells	african green monkey kidney cells	ATCC nr.: CRL-1586 TM

2.1.2 Hantavirus strains

Virus strain	Description	Source
HTNV (strain76-118)	pathogenic hantavirus, Asia	kindly provided by Âke Lundkvist, Stockholm
TULV (strain Moravia)	rather nonpathogeic hantavirus, Europe	kindly provided by Âke Lundkvist, Stockholm
PHV (type 3571)	rather nonpathogeic hantavirus, USA	kindly provided by Robert Tesh, Galvestone

2.1.3 Chemicals

Product	Manufacturer
acetone	Roth (Karlsruhe, Germany)
APS (ammonium persulfate)	Roth (Karlsruhe, Germany)
BME (basal medium with Earls's salt) 10x	Biochrom AG (Berlin, Germany)
bromphenol blue	Serva (Heidelberg, Germany)

Product	Manufacturer
BSA (bovine serum albumin)	PAA (Pasching, Austria)
DMSO (Dimethylsulfoxid)	Roth (Karlsruhe, Germany)
EMEM (minimal essential medium)	PAA (Pasching, Germany)
ethanol	Roth (Karlsruhe, Germany)
FCS (fetal calf serum)	Biochrom AG (Berlin, Germany)
Ficoll-Hypaque	PAA (Pasching, Austria)
formaldehyde	Merck (Darmstadt, Germany)
glycine	Roth (Karlsruhe, Germany)
GM-CSF (granulocyte-macrophage colony-stimulating factor)	Immunotools (Friesoythe, Germany)
HBSS (hanks' balanced salt solution)	PAA (Pasching, Austria)
HCl	Roth (Karlsruhe, Germany)
HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid)	PAA (Pasching, Austria)
L-glutamine	PAA (Pasching, Austria)
mercaptoethanol	Merck (Darmstadt, Germany)
methanol	Roth (Karlsruhe, Germany)
milk powder	Sufocin (Zeven, Germany)
PBS (phophat buffered saline)	PAA (Pasching, Austria)
penicillin/streptomycin (1000 U (units)/ml)	PAA (Pasching, Austria)
PGE1 (prostaglandin)	Sigma Aldrich (Deisendorf, Germany)
Poly-L-Lysine	Biochrom (Berlin, Germany)
RNaseA	Qiagen (Hilden, Germany)
Rotiphorese-Acrylamide	Roth (Karlsruhe, Germany)
RPMI Medium	PAA (Pasching, Austria)
SDS	Merck (Darmstadt, Germany)
staurosporine	Sigma-Aldrich (Deisendorf, Germany)
TEMED	ICN Biomedicals (Irvine, USA)
Tris-HCl	Roth (Karlsruhe, Germany)
thrombin	Sigma-Aldrich (Deisendorf, Germany)
triton X-100	Roche (Mannheim, Germany)
trypan blue	Serva (Heidelberg, Germany)

Product	Manufacturer
trypsin/EDTA	Invitrogen (Karlsruhe, Germany)
tween 20	Merck (Darmstadt, Germany)
PMA (4-β–phorbol-12-myristate-13-acetate)	Sigma-Aldrich (Deisendorf, Germany)
fibrinogen	Invitrogen (Karlsruhe, Germany)
fibronectin	Invitrogen (Karlsruhe, Germany)
vitronectin	Sigma Aldrich (Deisendorf, Germany)
ultra pure water	PAA (Pasching, Germany)

2.1.4 Equipment

Product	Manufacturer
autoclave	Getinge (Rastatt, Germany)
balance	Sartorius (Göttingen, Germany)
blockheater	Roth (Karlsruhe, Germany)
CCD-Camera	Bioblock Scientific (Illkirch, France)
CCD-Camera (L3-lab)	Raytest (Strabenhardt, Gemany)
centrifuge	Megafuge 2.0 R (Hanau, Germany)
CO_2 Incubator	Heraeus (Kleinostheim, Germany)
convocal laser scanning microscope	Nikon Instruments Europe B.V. (Amstelveen, the Netherlands)
counting chamber, Neubauer Improved	Marienfeld (Lauda-Königshofen, Germany)
cryo container	Nalgene Nunc (Wiesbaden, Germany)
electron microscope (TEM 902)	Carl Zeiss SMT AG
electrophoresis system Mini-Protean III	Bio-Rad (München, Germany)
ELISA reader	Biochrom Anthos (Cambridge, UK)
FACScalibur	Becton Dickinson (Heidelberg, Germany)
fluorescence microscope	Olympus (Hamburg, Germany)
Laminar flow box Herasafe	Heraeus (Kleinostheim, Germany)
microscope, Axiovert 25	Carl Ziess AG (Jena, Germany)
pH-meter pH 320	WTW (Weilheim, Germany)
refrigerator	Liebherr (Oberhausen, Germany)
semi-dry blot chamber	OWI (Portsmouth, Netherlands)
slow-scan-charge-coupled-device camera	Pro Scan (Scheuring, Germany)

Product	Manufacturer
ultramicrotome (Ultracut S)	Leica (Wetzlar, Germany)
vortexter Reax 2000	Reax Heidolph (Schwabach, Germany)
water bath	Lauda (Königshofen, Geramny)
whatman paper	Schleier & Schuell GmbH (Dassel, Germany)

2.1.5 Consumables

Product	Manufacturer
6-, 12-, 24-, 96-well plates	Nunc (Wiesbaden, Germany)
cell culture flask: T25, T75, T125	Nunc (Wiesbaden, Germany)
cover slides	Roth (Karlsruhe, Germany)
eppendorf tubes	VWR (Darmstadt, Germany)
FACS tubes	VWR (Darmstadt, Germany)
falcon tubes	VWR (Darmstadt, Germany)
LS-column	Miletnyi (Bergisch Gladbach, Germany)
microscope slides	Roth (Karlsruhe, Germany)
whatman paper	Schleicher & Schuell (Dassel, Germany)
pipet tips	Roth (Karlsruhe, Germany)
serological pipets	TPP (Trasadingen, Switzerland)
PVDF membrane	Milipore (Schwalbach/Ts., Germany)
sterile filters	Schleicher und Schüll (Dassel, Germany)
uncoated 96-well plates	Sarstedt (Nürnberg, Germany)

2.1.6 Primary monoclonal antibodies and polyclonal serum

Specificity	Species	Application	Manufacturer
β-Actin	mouse	WB	Acris (Herford, Germany)
CD14	mouse	FACS	BD Bioscience (München, Germany)
CD29	mouse	FACS, IF	ImmunoTools (Friesoythe, Germany)
CD41	mouse	FACS, IF	ImmunoTools (Friesoythe, Germany)
CD42	mouse	FACS, IF	ImmunoTools (Friesoythe, Germany)
CD51	mouse	FACS, IF	ImmunoTools (Friesoythe, Germany)
CD55	mouse	FACS	Southern Biotechnology Associates, (Birmingham, USA)

Specificity	Species	Application	Manufacturer
CD61	mouse	WB	BD Bioscience (München, Germany)
CD61	mouse	FACS, IF	ImmunoTools (Friesoythe, Germany)
CD62P	mouse	FACS	ImmunoTools (Friesoythe, Germany)
CD63	mouse	FACS	BD Bioscience (München, Germany)
CD69	mouse	FACS	BD Bioscience (München, Germany)
CD83	mouse	FACS	BD Bioscience (München, Germany)
CD86	mouse	FACS	BD Bioscience (München, Germany)
CD209 (DC-SIGN)	mouse	FACS	BD Bioscience (München, Germany)
gC1qR	mouse	FACS	Upstate Biotechnology (NY State, USA)
Hantavirus N-protein	rabbit	WB, IF	(Razanskiene *et al.*, 2004)
ICAM-I	mouse	FACS	BD Bioscience (München, Germany)
IgG1	mouse	FACS, IF	BD Bioscience (München, Germany)
IgG2b	mouse	FACS, IF	ImmunoTools (Friesoythe, Germany)
MxA	mouse	WB	(Flohr *et al.*, 1999)
RIG-I	rabbit	WB	Alexis (Lörrach, Germany)

2.1.7 Secondary antibodies and fluorescent dyes

Antibody	Species	Application	Manufacturer
AnnexinV	-	FACS	Santa Cruz Biotechnology (Heidelberg, Germany)
DAPI	-	IF	Invitrogen (Karlsruhe, German)
FITC (fluorescein isothiocyanate)	mouse	FACS, IF	Dianova (Hamburg, Germany)
HRP	mouse	WB	Dako (Glostrup, Denmark)
HRP	rabbit	WB	Dako (Glostrup, Denmark)
PE (phycoerithrin)	mouse	FACS	Dianova (Hamburg, Germany)
Propidiumiodide (PI)	-	FACS	Santa Cruz Biotechnology (Heidelberg, Germany)
Rhodamine-Phalloidine	-	IF	Invitrogen (Karlsruhe, Germany)
flouorescent fibrinogen	-	FACS	Invitrogen (Karlsruhe, German)
Texas Red	mouse	IF	Dianova (Hamburg, Germany)
TexasRed	rabbit	IF	Dianova (Hamburg, Germany)

2.1.8 Kits

Kit	Manufacturer
EZ4U Cell Proliferation Assay	Biomedica (Austria)
Monocyte Isolation Kit II	Miltneyi Biotec (Bergisch Gladbach, Germany)
In Situ Cell Death Detection Kit	Roche (Mannheim, Germany)
Super-Signal® West Dura Extended Duration Substarte	Thermo Scientific (USA, Rickford)
Venor® GeM-Mycoplasma Detection Kit	Minerva Biolabs (Berlin, Germany)

2.1.9 Software

Software	Manufacturer
Adobe Photoshop	Adobe Systems Incorporated (München, Germany)
CellQuest Pro	Becton Dickinson (Heidelberg, Germany)
Corel Draw	Corel Corporation (Unterschleißheim, Germany)
PRISM	GraphPad Software Inc. (La Jolla, USA)
TINA	Raytest (Straubenhardt, Germany)

2.1.10 Buffers and Solutions

Buffer/ Solution	Ingredients
0,1% triton	0.1% triton on PBS
10% resolving gel	3.3 ml 30% acrylamide/0.8% bisacrylamide (37:55:1), 2.5 ml 1.5 M Tris-HCl, pH 8.8, 100 µl 10% SDS, 100 µl 10% APS, 6 µl TEMED, 4ml H_2O
2x BME	100 ml 10x BME, 10 ml L-glutamine, 40 ml $NaHCO_3$, 350 ml H_2O
4% formaldehyde solution	4% Formaldehyde in PBS
acetone/methanol	acetone : methanol, 1:1
AnnexinV Buffer	10 nM HEPES, 0.14 M NaCl, 5 mM CaCl2, 5% FCS, 0.02% sodium acid in H_2O
antibody dilution buffer	PBS, 5% FCS, 0.1% tween
blocking solution	PBS, 1% FCS
buffycoat medium	RPMI, 0.2mM EDTA
coating buffer	PBS, 0.5% BSA

Buffer/ Solution	Ingredients
FACS block	PBS, 10% FCS, 0.2% sodium acid
FACS fixation solution	PBS, 1% formaldehyde
FACS wash	PBS, 1% FCS, 0.02% sodium acid
fixation buffer (electron microscopy)	2.5% Gutaraldehyde in HEPES
fixation buffer II (electron microscopy)	1% OsO_4, 1% uranyl acetate and 0.1% tannic acid in HEPES-buffer
HEPES Tyrodes buffer	137 mM NaCl, 2.9 mM KCl, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM glucose, 10 mM HEPES
overlay medium	1:1 mixture of : 1% agarose: 2x BME, 10% FCS, 2.5% HEPES, 100 IU/ml penecillin, 100 IU/ml streptomycin
PBS	8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24g KH_2PO_4, ad 1l H_2O
protein lysis buffer	250 mM tris, 2% SDS, 10% glycerine, 5% mercaptethanol, 0.01% bromphenol blue, in H_20
SDS buffer	60 g Tris, 288 g Glycin, 20 g SDS, H_2O ad 2l
semi dry buffer	200 ml methanol, 1.87 ml 20% SDS solution, 5.2 g Tris, 2.425 g tris, H_2O ad 1 l
stacking gel	0.83 ml 30% acrylamide/0.8 % bisacrylamide (37:55:1), 1.25 ml 0.5 M tris-HCl, pH 6.8, 50 µl 10% SDS, 50 µl 10% APS, 6 µl TEMED, 2.8 ml H_2O
stripping solution	100 mM tris, 2% SDS, 0.1% mercaptoethanol
virus dilution buffer	Hank´s balanced salt solution, 2% FCS, 100 IU/ml penecillin, 100 IU/ml streptomycin, 2% HEPES
washing buffer	PBS, 0.15% tween

2.2 Methods

2.2.1 Cell Biological Methods

2.2.1.1 Cell Culture

HEL, K562 and Meg-01 cells were maintained in RPMI medium supplemented with 10% FCS, 2mM L-glutamine, penicillin and streptomycin. Vero E6 cells were grown in minimal essential medium (MEM) supplemented with the same additives. TF-1 cells were maintained in RPMI medium, supplemented with 2 mM L-glutamine, 10% FCS and 5 ng/ml recombinant human GM-CSF. L8057 cells were maintained in RPMI, 10% FCS and 2 mM L-glutamine. Platelets were cultured in Hepes-Tyrodes Buffer. Monocytes and Lymphocytes were cultured in RPMI medium supplemented with 10% FCS, 100 mM Hepes, 2mM L-glutamine, penicillin and streptomycin.

2.2.1.2 Stimulation of cells

HEL cells or Vero E6 cells were stimulated with 10 nM PMA (4-β–phorbol-12-myristate-13-acetate) 1 day before or 1 hour (h) after infection.

2.2.1.3 Isolation of platelets

Platelets were isolated from freshly drawn citrated blood. The first 2 ml of blood were discarded. Blood was centrifuged at 80 g for 15 min. After centrifugation platelet rich plasma (prp) was pipetted off and 1 µl PEG1/ml was added to platelet rich plasma. After further centrifugation (200 g, 5 min) platelets were concentrated. Isolated platelets were resuspended in Hepes-Tyrodes buffer. For stimulation 0.2 U (units)/ml thrombin were added.

2.2.1.4 Determining cell count

For cell counting, cells were diluted with a 0.5% trypan blue solution to differentiate between living and dead cells as trypan blue stains only dead cells. 10 µl of the cell dilution were filled evenly at once to hematocytometer chamber. Cells were allowed to settle down for two minutes. Cells in one big square (16 small squares) were counted and cell number was determined by following formula:

$$\text{cells x dilution factor x } 10^4 = \text{cells/ ml}$$

For counting of platelets, cells on 80 squares of the middle of the counting chamber were counted. Cell number was verified by following formula:

$$\frac{\text{platelets x dilution factor}}{0.02} = \text{platelets/ µl}$$

2.2.1.5 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from buffy coat preparations derived from healthy human donors. Blood was diluted with buffy coat medium 1:2. The mixture was layered on Ficoll-Hypaque and centrifuged (20 min, 800 g, room temperature, no brake). Subsequently PBMCs, concentrated in a white layer on Ficoll-Hypaque, were collected and washed with medium. To remove residual platelets from PBMCs, cells were washed for a second time.

2.2.1.6 Isolation of monocytes

Monocytes were isolated from PBMCs by using the Monocyte Isolation Kit II, according to the manufacturer's protocol. In brief, PBMCs were diluted in 10 ml medium and layered on diluted Ficoll-Hypaque (1.4 ml in PBS). After centrifugation (800 g, 25 min, room temperature, no brake) PBMC layer was harvested. Cells were washed with ice-cold MACS buffer and blocked for 10 min at 4°C with FcR blocking reagent and 200 µl of monocytes-Biotin-Antibody-Cocktail (containing antibodies directed against anti-human CD3, CD7, CD16, CD19, CD56, Glycophorine A). Thus, non-monocytic cells were labelled. To deplete labelled cells, 400 µl Anti-Biotin Micro Beads were added. After 15 min of further incubation cells were adjusted to an equilibrated LS column. Monocytes were collected in flow through.

2.2.2 Virological Methods

2.2.2.1 Safety aspects

Working with pathogenic hantaviruses has to be performed under laboratory safety 3 conditions. This implies performing all infectious work in a special laboratory with low pressure and wearing protective clothing. Handling viable virus has to be performed

under laminar flow box exclusively. All kinds of waste must be autoclaved before being discarded.

2.2.2.2 Virus stock production

Virus stocks of HTNV, Tula and PHV were propagated on VeroE6 cells. Medium of cells of a T75 flask was taken off. Cells were infected with 500 µl stock and kept on a shaker at 37°C for one h. Afterwards 25 ml of Medium was added and cells were kept in a humidified atmosphere at 37°C for 7-10 days. Culture supernatant was harvested, 5% inactivated FCS added, aliquoted and stored at –80°C. To increase viral titers cell culture supernatant of infected VeroE6 cells was concentrated in an ultracentrifuge at 130,000g at 4°C for 3 h. For infection of platelets virus stocks were ultracentrifugated and virus resuspended in Hepes-Tyrodes Buffer. Virus stocks were tested for mycoplasma by polymerase chain reaction (PCR) regularly.

2.2.2.3 Virus infection of cells

Virions at respective multiplicity of infection (MOI) (0.1, 1.0 or 1.5) were allowed to adsorb to cells for 1 h. After washing three times with medium cells were seeded in a cell culture vessel and incubated in a humidified atmosphere at 37°C. Uninfected cells treated with medium instead of virions were used as mock control.

2.2.2.4 Virus titration

Hantavirus titers were determined as described recently (Heider *et al.*, 2001). Briefly, Vero E6 cells were infected by serial dilutions of supernatants for 1 h to allow adsorbance of virus particles. Thereafter, cells were overlaid with medium containing 0.5% agarose. After 7 days of incubation at 37°C the overlay was removed, cells were washed with PBS and fixed with methanol for 10 min. After incubation with a hantavirus N-protein-specific polyclonal rabbit serum the SuperSignal® West Dura Extended Duration Substrate kit was used according to the manufacturer's instructions to visualize antigen-positive foci. Finally, the antigen-positive foci were counted to calculate virus titers, which are expressed as FFU/ml.

2.2.2.5 Kinetics

To analyse virus growth in different cells growth kinetics were performed. Cells were infected and seeded to cell culture vessels in adequate concentration. At indicated time points supernatant was harvested. Viral titers were analysed by titration.

2.2.3 Molecular Biological Methods

2.2.3.1 Cell proliferation

For differentiating between dead and vital cells a 0.5% trypan blue solution was used. 10 µl of the cell dilution were filled into a hematocytometer chamber. Dead and live cells were counted and cell number was determined.

2.2.4 Electron microscopy

Cells were fixed in fixation buffer for 1 h at room temperature and centrifuged. Pellets were embedded in agar blocks by mixing equal volumes of cell-pellet and low-melting-point agarose. Small agar blocks were cut to size and postfixed with fixation buffer II. Samples were dehydrated stepwise in graded ethanol series and embedded in epon resin, which was subsequently polymerized. Thin sections were prepared with an ultramicrotome and counterstained with 2% uranyl acetate and lead citrate. The sections were examined using a TEM 902 at 80 kV. Obtained images were digitized using a slow-scan charge-coupled-device camera.

2.2.5 Proteinchemistry

2.2.5.1 Immunoblot

HEL cells were lysed in lysis buffer followed by denaturation by heating the sample for 5 min at 95°C. Proteins were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. Subsequently membrane was blocked in 5% milk powder for one hour. The membrane was incubated with first antibody at 4°C over night. For detection of primary antibodies an anti-rabbit or anti-mouse peroxidase-conjugated antibody was used. Immunoblots were developed by using SuperSignal® West Dura Extended Duration Substrate kit according to the manufacturer's protocol.

2.2.6 Immunological Methods

2.2.6.1 Immunofluorescence

Coverslides were washed twice with water and sterilized with 70% ethanol. After drying, cover slides were coated with poly-l-lysine, incubated at 37°C for 30 min and washed twice with water. Cells were harvested and washed three times, resuspended in medium and allowed to adhere to poly-l-lysine coated coverslides for 15 min at 37 °C. Cells were either fixed with acetone/methanol for 10 min or with 4% formaldehyde for 60 minutes. Formaldehyde fixed cells were permeabilized with 0.1% Triton for 5 min and washed in PBS three times. Before staining cells were blocked in blocking solution for 30 min at 4°C followed by three washing steps in PBS. First antibody was diluted in dilution buffer. Coverslides were incubated at 37°C in a moist chamber in the dark for one hour. For detection FITC or Texas Red labelled secondary antibodies (1:400) were used.

2.2.6.2 Flow cytometry

For surface staining cells were harvested and washed twice in ice-cold FACS washing solution. Thereafter, cells were resuspended in 50 µl FASC blocking solution, containing the primary antibody in appropriate dilution, and incubated for 1 h. After incubation, cells were again washed twice with FACS wash and for unconjugated primary antibodies secondary antibody, diluted in FACS block solution, was added. After 45 min the cells were washed with FACS wash solution and resuspended in FACS fixation solution. For quantifying fluorescence of labelled cells a FACSCalibur® was used. Results were evaluated with the flow cytometry analysis software program CellQuestPro®.

2.2.6.3 Endoreplication

For analysis of endoreplication HEL cells were harvested and adjusted to $1x10^6$ cells/ml. After fixation in ice-cold 70% ethanol cells were washed with PBS. To eliminate RNA, RNA was digested with RNase I (50 µg/ml) for 30 min. Propidiumiodide was added 20 min prior to FACS analysis at 50 µg/ml.

2.2.6.4 Analysis of cell death by terminal-desoxynucleotidyl-transferase-mediated 2-Deoxyuridine 5'-Triphosphate (dUTP) nick end labeling (TUNEL) technology

To detect cells death via DNA strand breaks TUNEL technology was used according to manufacturer's protocol (*In Situ* Cell Death Detection Kit, Roche). Briefly, cells were fixed in 4% formaldehyde on coverslides. Cells were permeabilized with 0.1 % triton solution and TUNEL reaction mixture was added. Cells were analysed by confocal laserscan microscopy.

2.2.6.5 Analysis of cell death by flow cytometry

For quantification of apoptosis cells were harvested, washed once with AnnexinV buffer and incubated with FITC-labelled AnnexinV for 20 min at 4°C. Cells were washed again with AnnexinV buffer and fixed in fixation buffer. As a positive control cells were incubated with staurosporine (0.5 µg/ml) over night. Finally cells were analysed by flow cytometry as described before.

2.2.6.6 Adhesion assay

To test adhesion capacities of HEL cells uncoated 96-well plates were coated with 10 µg/ml fibrinogen, 10 µg/ml fibronectin or 0.5 µg/ml vitronectin at 4°C overnight. After washing off ligands three times with PBS, wells were coated with coating buffer for 60 min at 37 °C. HEL cells that had been infected with HTNV and harvested three days post infection, were adjusted to $5x10^4$ cells/well and allowed to adhere to ligands for 90 minutes. Non-adherent cells were washed off three times with PBS. The number of adherent cells was quantified by using a colorimetric assay (MTT assay) according to manufacturer's protocol. The assay is based on the fact that living cells metabolize tetrazolium salt into its deep red coloured formazan derivative. For quantification of adherent HEL cells, cells were covered with 200 µl cell culture medium and 20 µl of dye substrate. Optical density (OD) was recorded after 3 h at 450 nm. Experiments were done in triplicates.

2.2.7 Internalisation assay

For the analysis of receptor internalisation cells were infected at MOI 1.5 three days pi. Cells were stained for CD29 or CD61 in culture medium for 1 h. After being washed twice, cells were incubated for 2 h at either 4°C or 37°C. Subsequently, cells were stained for remaining cell surface-bound antibody with a secondary antibody. The reduction in specific staining at 37°C compared to the specific staining at 4°C represents the level of internalisation.

2.3 Statistical methods

The Wilcoxon test was used to analyse inter-group differences. A value of $p<0.05$ was considered significant (two tailed). Box-Whisker plots cover data values between 10th-90th percentiles; the central line indicates the median. Endpoints of whiskers represent true data points for smallest and biggest values.

3 Results

3.1 Hantaviruses and cells of the haemostatic system

Megakaryocytes are myeloid cells that arise from haematopoietic stem cells in the bone marrow. Several steps of differentiation and maturation are required until the mature megakaryocytes are formed (megakaryopoiesis). These cells further develop into proplatelets that are released into the blood. Subsequently platelets are produced by cytoplasmic fragmentation (thrombopoiesis). Platelets are primarily known for their pivotal function in blood clotting, but are also important immune cells.

Acute thrombocytopenia during hantavirus infection represents an important factor in the cascade of events leading to haemorrhages. Surprisingly, megakaryocytes and platelets have not been of prime interest in the field of hantaviral pathogenesis so far. In 1977 megakaryocytes of patients suffering from Korean haemorrhagic fever were analysed by light and electron microscopy (Lee *et al.*, 1977). They found that in 12 out of 14 patients platelet formation by megakaryocytes was absent. Furthermore in 2004 Liang and coworkers detected hantavirus antigen in 20% of megakaryocytes in the bone marrow of HTNV-infected patients (Liang *et al.*, 2004).

In order to explore the mechanisms underlying acute thrombocytopenia and haemorrhages during hantavirus infection, we analysed the interaction of hantaviruses with cells of the haemostatic system: human megakaryocytes and platelets. Hantaviruses might interfere at different developmental stages with megakaryopoiesis and thrombopoiesis (figure 5). Infection or simply interaction of the virus with differentiating megakaryocytes could impair cellular function on several stages and/or formation of platelets. In addition, hantaviruses could modulate the function of platelets.

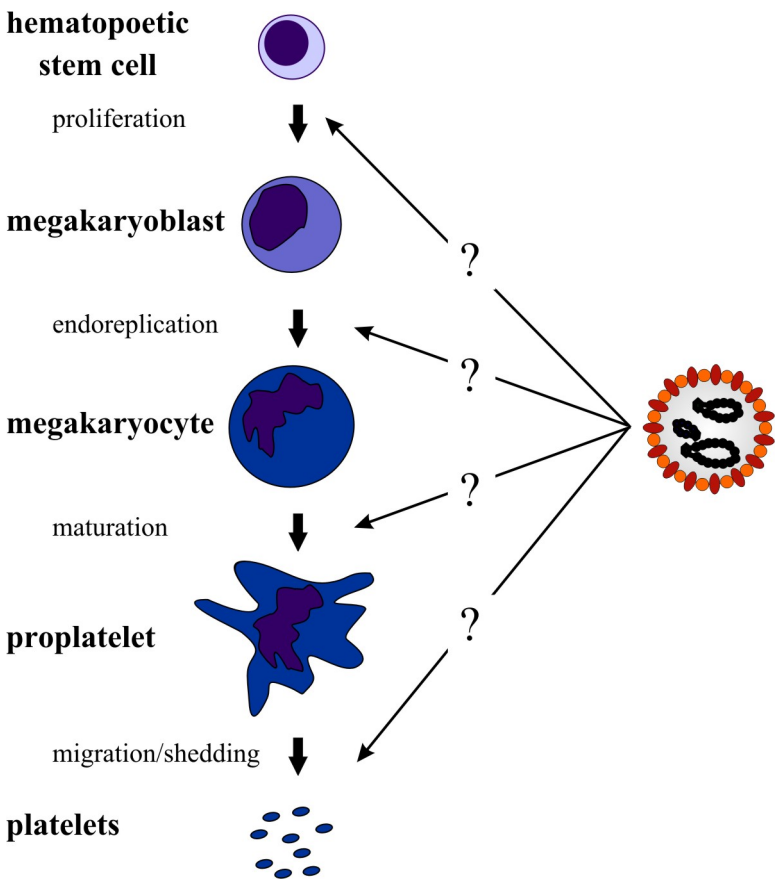

Figure 5: Simplified scheme of megakaryopoiesis and thrombopoiesis.
Thin arrows demonstrate possible sides of interaction between cells and viruses.

3.2 Megakaryocytic cell lines as a model for megakaryopoiesis

Isolation and cultivation of primary megakaryocytes is very difficult to achieve. Therefore, megakaryocytic cell lines serve as model systems to analyse megakaryopoiesis *in vitro*. In this study the human megakaryocytic cell lines TF-1, HEL, K562, Meg-01 that are arrested at different stages of differentiation and the murine megakaryocytic cell line L8057 were used. HEL, Meg-01, K562 and L8057 cells further differentiate along the megakaryocyte lineage after stimulation with low doses of PMA (Alitalo, 1990; Isakari *et al.*, 2009; Long *et al.*, 1990). TF-1 cells undergo terminal megakaryocytic differentiation when stimulated with PMA and TPO (Testa *et al.*, 1998), but apoptosis is induced simultaneously (Chao *et al.*, 1997). In this study, therefore, only unstimulated TF-1 cells were analysed.

TF-1 is a premyeloid cell line originally isolated from bone marrow of a patient suffering from erythroleukemia. It represents a less differentiated cell type (Kitamura *et al.*, 1989). HEL cells, isolated from peripheral blood of a 30-year-old man with

erythroleukemia, and K562 cells, isolated from the pleural effusion of a 53-year-old woman suffering from chronic myeloid leukemia in blast crisis, represent early megakaryoblast stages (Lozzio & Lozzio, 1975; Martin & Papayannopoulou, 1982). Meg-01 cells were established from the bone marrow of a 55-year-old man with chronic myeloid leukemia in megakaryocytic blast crisis, and also originate from the megakaryocytic lineage (Ogura *et al.*, 1985). L8057 cells are murine megakaryoblast cells isolated from an irradiated C3H/He mouse (Ishida *et al.*, 1993).

Of all cells used during these experiments TF-1 cells represent the most undifferentiated stage. K562 and HEL cells comprise erythroid as well as megakaryocytic markers. It is assumed that they are derived from a haematopoietic progenitor cell that gives rise to both cell types (Gewirtz *et al.*, 1982; Martin & Papayannopoulou, 1982). HEL cells carry markers that are already expressed by undifferentiated megakaryocytes whereas K562 cells only commit to the megakaryocytic lineage after stimulation with PMA. Meg-01 cells show many megakaryocytic characteristics but also express CD14 and BA-1 as non-megakaryocytic proteins. They are known to produce platelet-like particles (Ogura *et al.*, 1985; Takeuchi *et al.*, 1991). Figure 6 shows surface expression of markers of megakaryopoiesis on the human cell lines used in this study.

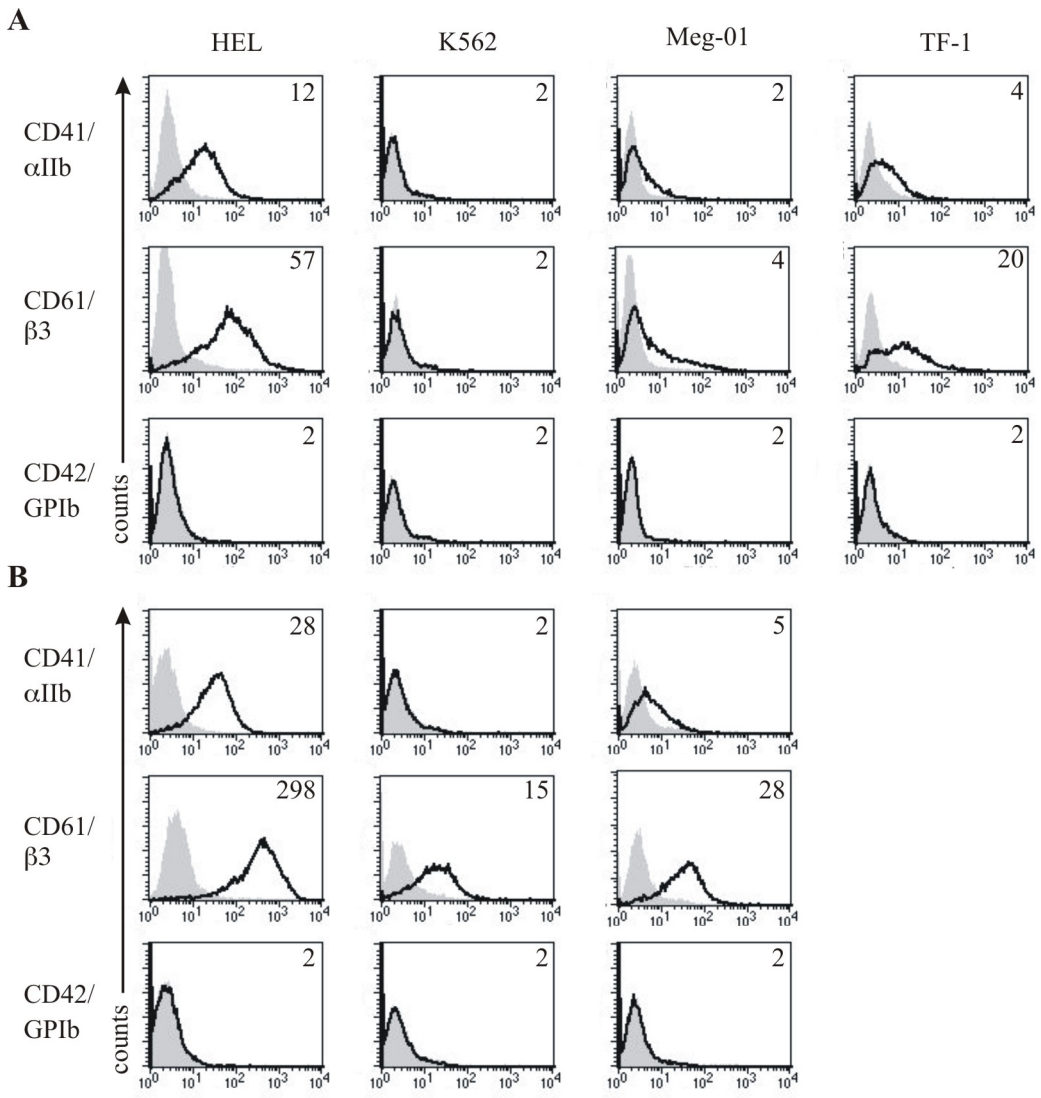

Figure 6: Expression of markers of megakaryopoiesis on unstimulated (A) and PMA-stimulated (B) human megakaryocytic cell lines.

Unstimulated cells (A) and cells stimulated with 10 nM PMA (B) were analysed for CD41 (integrin αIIb), CD61 (integrin β3), and CD42 (GPIb) by flow cytometry. Grey filled histograms represent isotype controls, black histograms show specific staining with accordant primary antibody. On x-axis fluorescence intensity, on y-axis cell count is indicated. Numbers in the right corner of histograms indicate mean fluorescent intensity. Data shown are representatives of three independent experiments

Analysis of typical surface markers of megakaryocytic cells showed, that different cell lines express on the surface markers of megakaryopoiesis with different densities. TF-1 cells represent a very early phase within megakaryopoiesis. Late megakaryopoiesis marker CD42 is not expressed at all. However, low expression of CD41 and CD61 was detected. Unstimulated K562 cells express hardly any marker protein, but upon stimulation with PMA expression of CD61 is increased, indicating terminal differentiation along the megakaryocytic lineage. Untreated HEL cells express

CD41 and CD61 on their surface, but no CD42. Stimulation leads to an increased expression of both early markers, especially CD61. Meg-01 cells only express low amounts of CD41 and CD61 when not stimulated with PMA. After stimulation expression of both markers is slightly increased.

3.3 Hantavirus receptors on megakaryocytic cells

For hantaviruses different receptors for viral entry have been detected. Highly pathogenic hantaviruses enter cells via β3 integrins whereas rather non-pathogenic hantaviruses use β1 integrin for entry (Gavrilovskaya *et al.*, 1999; Gavrilovskaya *et al.*, 2002). Furthermore, decay-accelerating factor CD55/DAF and the receptor for globular head domain of complement component C1q (gC1qR) have been shown to mediate entry of HTNV into host cells (Choi *et al.*, 2008; Krautkramer & Zeier, 2008). To investigate which of these receptors are expressed on the surface of the megakaryocytic cell lines used, cells were analysed by flow cytometry (Figure 7: Surface expression of hantavirus receptors on human megakaryocytic cells. (Figure 7).

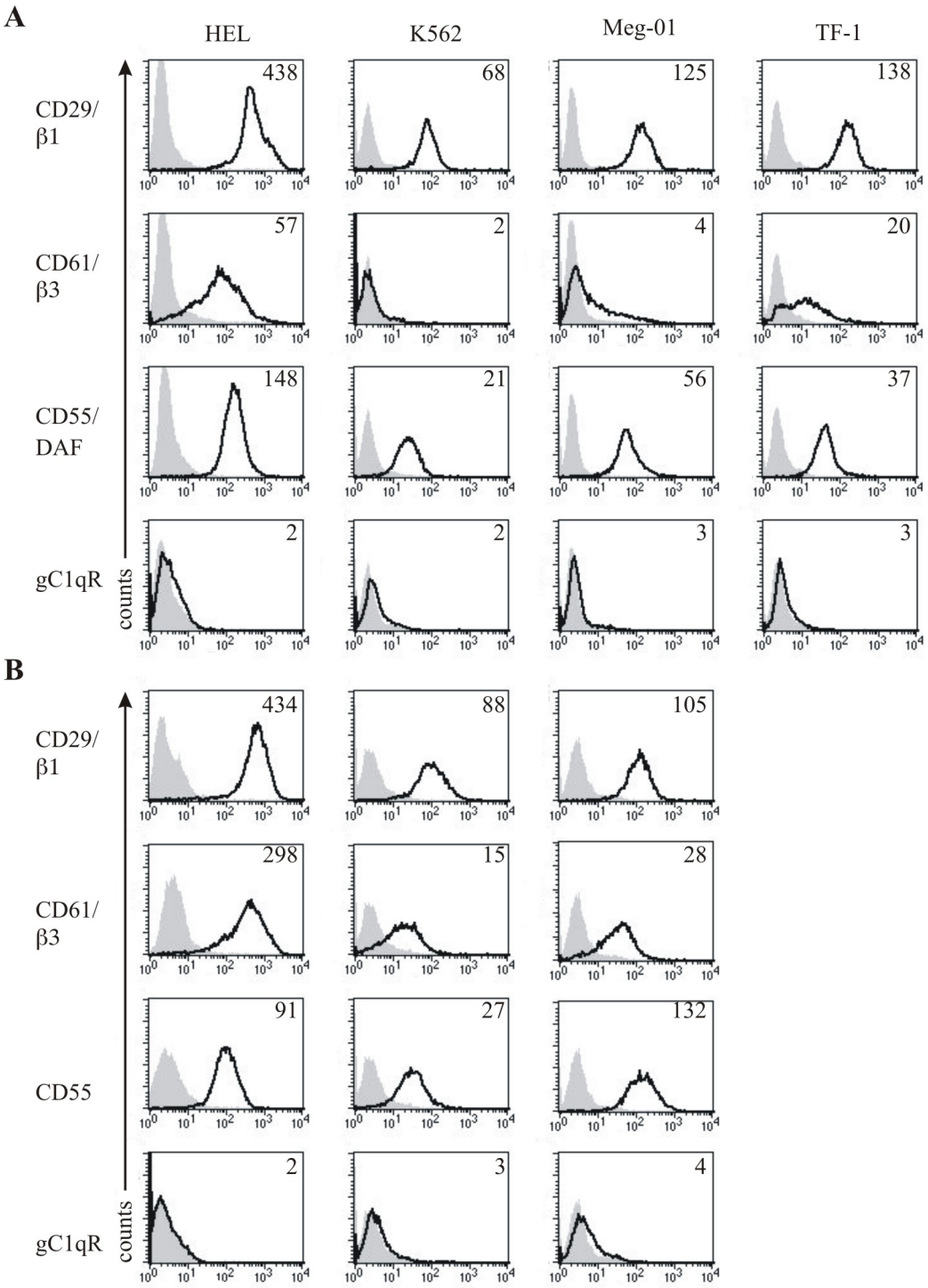

Figure 7: Surface expression of hantavirus receptors on human megakaryocytic cells.

Unstimulated cells (A) and cells stimulated with 10 nM PMA (B) were analysed for β1, β3, CD55/DAF and gC1qR by flow cytometry. Grey filled histograms represent isotype controls, black histograms show specific staining with accordant primary antibody. On the x-axis fluorescence intensity, on the y-axis cell count is indicated. Numbers in the right corner of histograms give the mean fluorescent intensity. Data shown are representatives of three independent experiments.

CD29 (β1) and CD55/DAF were strongly expressed on all cell lines analysed. When stimulated with PMA, expression of CD55/DAF was slightly decreased on HEL cells but increased on Meg-01 cells. Stimulation with PMA did not significantly alter CD29 (β1) expression. CD61 (β3) was already strongly expressed on unstimulated HEL cells, barely found on unstimulated Meg-01 and TF-1 cells, and undetectable on unstimulated K562 cells. After PMA-induced differentiation into megakaryocytes, expression of CD61 was strongly increased on all cell lines tested. In contrast to the other hantavirus receptors, gC1qR was found neither on unstimulated nor on PMA-stimulated megakaryocytic cells.

3.4 Infection of megakaryocytic cell lines with hantaviruses

3.4.1 Switch to high-level HTNV infection in differentiating HEL cells

Amongst the megakaryocytic cell lines tested HEL cells showed the strongest surface expression of hantavirus receptors. For this reason, the susceptibility of unstimulated and PMA-stimulated HEL cells to pathogenic HTNV and to the rather non-pathogenic hantavirus strains TULV and PHV was analysed by immunohistochemistry (figure 8).

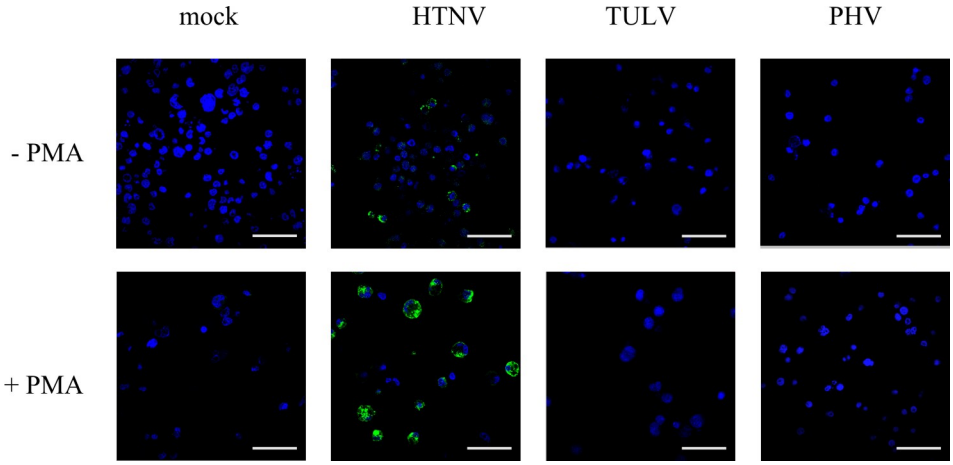

Figure 8: Immunofluorescence microscopy analysis of unstimulated and PMA-stimulated HEL cells infected with HTNV, TULV or PHV.

Unstimulated and PMA-stimulated HEL cells were infected with hantavirus strains as indicated and stained for viral N-protein using a rabbit-derived polyclonal antiserum (green) 3 days pi. Nuclei were stained with DAPI (blue). Original magnification x60, bar = 50 µm.

Indeed viral N-protein could be detected in HEL cells inoculated with HTNV. Intriguingly, after induction of HEL cell differentiation with PMA, expression of viral N-protein in HTNV-infected HEL cell cultures was increased and more widespread. In contrast, both unstimulated and PMA-stimulated cell cultures inoculated with PHV or TULV remained negative for viral antigen. Confocal laserscan microscopy confirmed the presence of viral N-protein in the cytoplasm of HTNV-infected HEL cells (figure 9A). In electron microscopy analysis HTNV-infected HEL cells showed the typical virus-induced intracytoplasmic granulofilamentous inclusion bodies (Figure 9B) that are closely associated with the rough endoplasmic reticulum (ER) and the ER-Golgi intermediate compartment (ERGIC) (Ramanathan *et al.*, 2007).

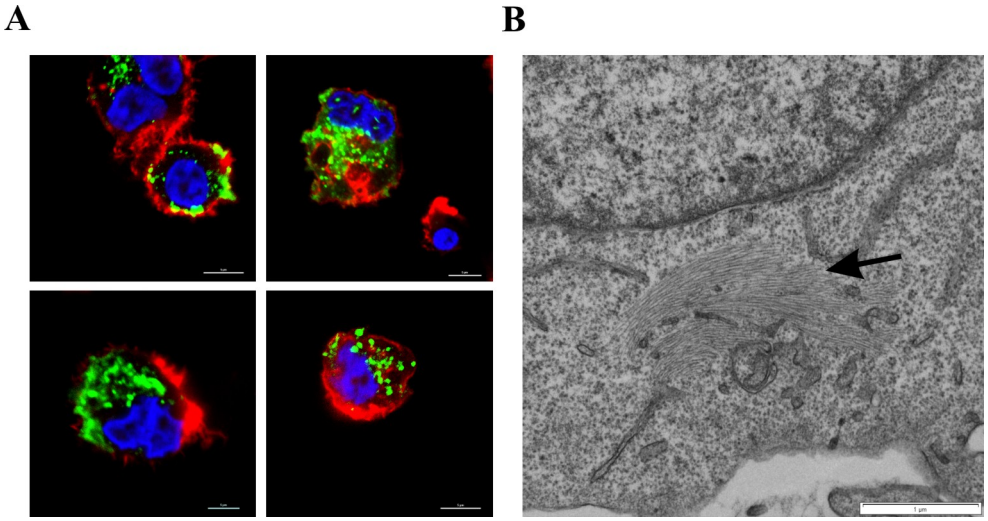

Figure 9: Analysis of HTNV-infected HEL cells by confocal laserscan- and electron microscopy.
(A) PMA-stimulated HEL cells were stained with antibody directed against viral protein (green) three dpi. Cells were stained against viral N-protein using a rabbit-derived polyclonal antiserum (green) and filamentous actin using rhodamine labelled phalloidine (red). To localize the nucleus, DNA was stained with DAPI (blue), bar = 5 µm. (B) Unstimulated HEL cells were infected with HTNV and analysed by electron microscopy three dpi. Typical filamentous structures of viral N-protein were detected in the cytoplasm of the cell (black arrow), bar = 1µm.

The growth pattern of HTNV in HEL cells was investigated in more detail. As shown in figure 10A HTNV replicated with moderate efficiency in undifferentiated HEL cells reaching titers of approximately 10^3 FFU/ml. Intriguingly, a switch from low-level to high-level virus production occurred after PMA-induced differentiation of HTNV-infected HEL cells. The titers increased more than 100 fold and reached peak titers between 10^5 and 10^6 FFU/ml. Figure 10B shows immunofluorescence picture of

unstimulated and PMA-stimulated HTNV-infected HEL cells. The different pattern of infection can be clearly seen. This strong increase in virion production was observed although PMA-induced differentiation of HEL cells was associated with an arrest in proliferation (figure 15, p. 43).

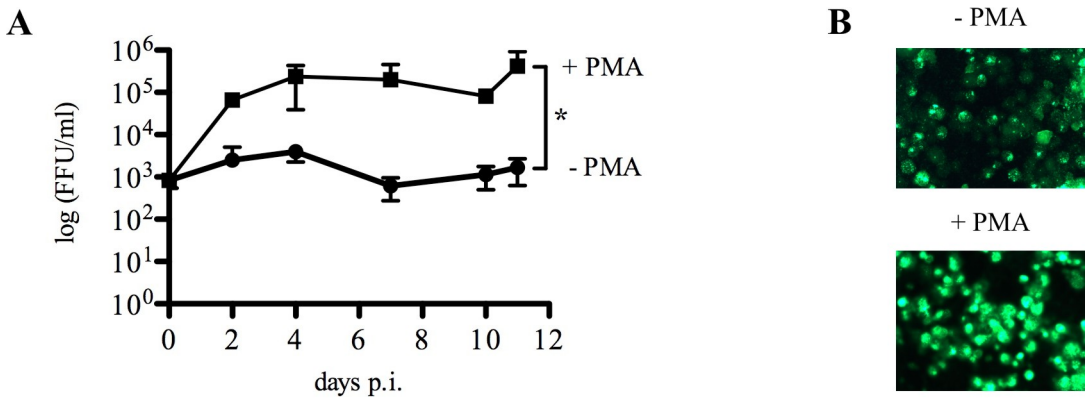

Figure 10: Infection and replication of HTNV in HEL cells.

(A) Growth kinetics of HTNV in unstimulated and stimulated HEL cells. Cells were infected with HTNV at MOI 1.5. Virus was allowed to adsorb to cells for one hour and washed three times with cell culture medium. Afterwards cells were resuspended in medium at a concentration of 500,000 cells/ml. Stimulation with PMA (10 nM) was performed right after infection. Supernatant was harvested at indicated time points. Results show means ±1 SD (standard deviation) out of three independent experiments. (B) Immunofluorescence of HEL cells infected with HTNV in presence and absence of PMA. Cells were harvested three days pi. Viral nucleoprotein was visualized by using a rabbit-derived polyclonal antiserum against viral N-protein (green). Original magnification x60.

Collectively, these data demonstrate that PMA-induced differentiation of HEL cells was associated with drastically upregulated release of HTNV virions.

3.4.2 Switch to high-level HTNV replication independent from PMA-induced upregulation of the HTNV receptor

Next we investigated whether PMA-induced upregulation of β3 integrin, the HTNV receptor on the surface of HEL cells, contributes to the switch to high-level HTNV replication after PMA treatment (figure 11A). After stimulation with PMA for one day, surface expression of β3 integrin is already increased on HEL cells. Therefore HEL cells were treated with PMA either one day before infection or directly after infection and virus titers were determined over time. Furthermore, it was analysed whether the

increased titers observed with HEL cells were due to stimulation of VeroE6 cells with residual PMA during titration. For this purpose, VeroE6 cells were infected with HTNV, stimulated with PMA, and supernatants were titrated (figure 11B).

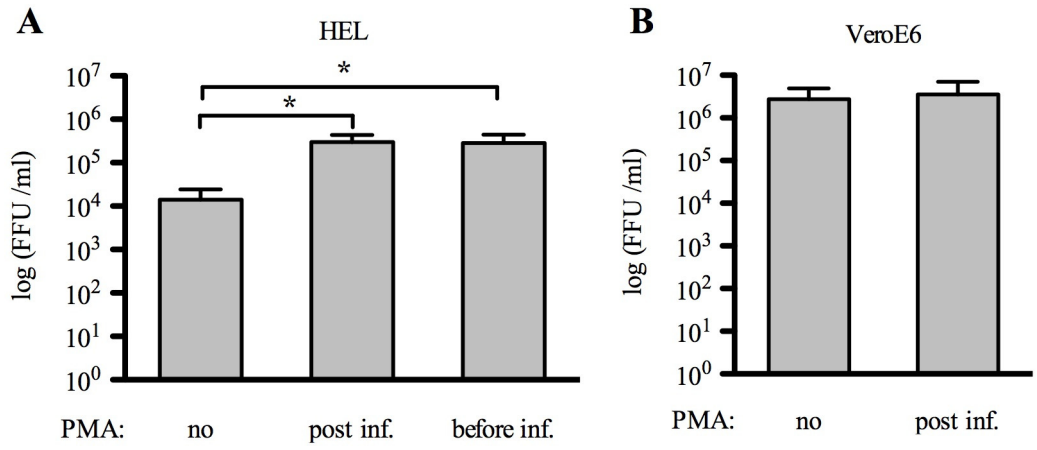

Figure 11: Titers of HTNV in HEL and in Vero E6 cells after PMA treatment.

(A) Titers of HTNV in the supernatant of HEL cells three days pi without PMA stimulation, with PMA stimulation (10 nM PMA) directly after infection or with PMA stimulation one day before infection (10 nM PMA). (B) Titers of HTNV in supernatant of VeroE6 cells three days pi without PMA stimulation or with PMA stimulation (10 nM PMA) directly after infection. ∗ $p < 0.05$.

Stimulation of HEL cells with PMA prior to infection did not increase viral output as compared to HEL cells stimulated with PMA directly after infection. Moreover, PMA stimulation of VeroE6 cells did not influence HTNV replication. In conclusion, increased titers of HTNV in PMA-stimulated HEL cells are caused by a direct effect of PMA on HEL cells, which is independent of PMA-induced upregulation of β3, and not by an indirect effect on VeroE6 cells during titration.

3.4.3 Infection of TF-1 cells

TF-1 cells express markers of the erythroid as well as of the megakaryocytic lineage similar to HEL cells. TF-1 cells were infected with HTNV, TULV and PHV at MOI 1.5 and analysed by immunofluorescence microscopy for infection (figure 12).

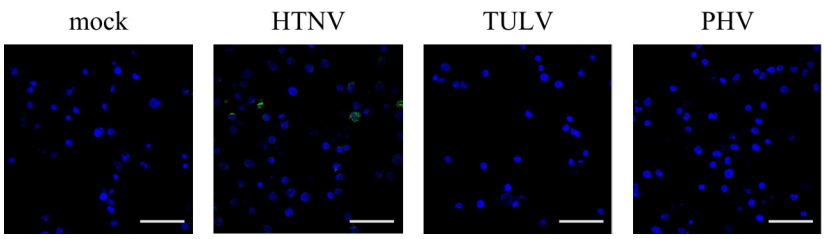

Figure 12: Immunofluorescence microscopy analysis of TF1 cells infected with HTNV, TULV and PHV.

Unstimulated TF-1 cells were infected with hantavirus strains as indicated and stained for viral N-protein using a rabbit-derived polyclonal antiserum (green) three days pi. Nuclei were stained with DAPI (blue). Original magnification x60, bar = 50 µm.

Similar to HEL cells viral N-protein was found in TF-1 cells after HTNV infection but not after infection with TULV and PHV.

The growth pattern of HTNV in TF-1 cells was analysed in more detail. For this purpose, supernatant of HTNV-infected TF-1 cells were collected at different time points after infection and the titer was determined (figure 13).

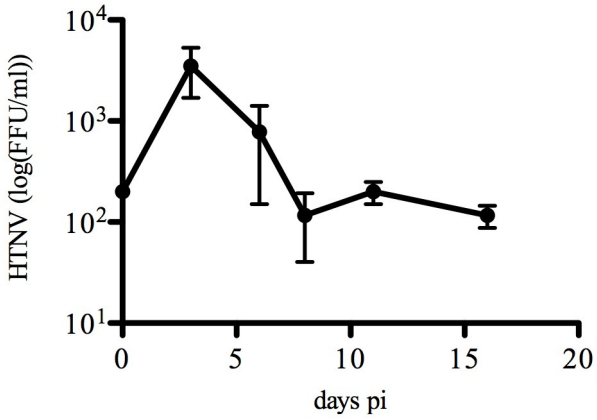

Figure 13: Growth kinetics of HTNV in TF-1 cells.

TF-1 cells were infected with HTNV at MOI 1.5. Virus was allowed to adsorb to cells for one hour followed by three times washing with cell culture medium. Cells were seeded at a concentration of 500,000 cells/ml. Supernatant was harvested at indicated time points and analysed by titration. Results are means ±1 SD derived from three independent experiments.

The peak virus titer was observed three days after infection. Until day 8 pi the output of viral particles decreased by two logs and then stayed constant until 16 days pi.

3.4.4 Resistance of K562, Meg-01 and L8057 cells to hantavirus infection

In addition to HEL and TF-1 cells further cell lines of the megakaryocytic lineage were used for infection experiments: the human megakaryocytic cell lines K562 and Meg-01 (described in 3.2) and, since rodents are natural reservoir hosts for most hantaviruses, the murine megakaryocytic cell line L8057. Cells were infected with HTNV at MOI 1.5 and analysed by immunohistochemistry for the presence of viral N-protein three days pi (figure 14).

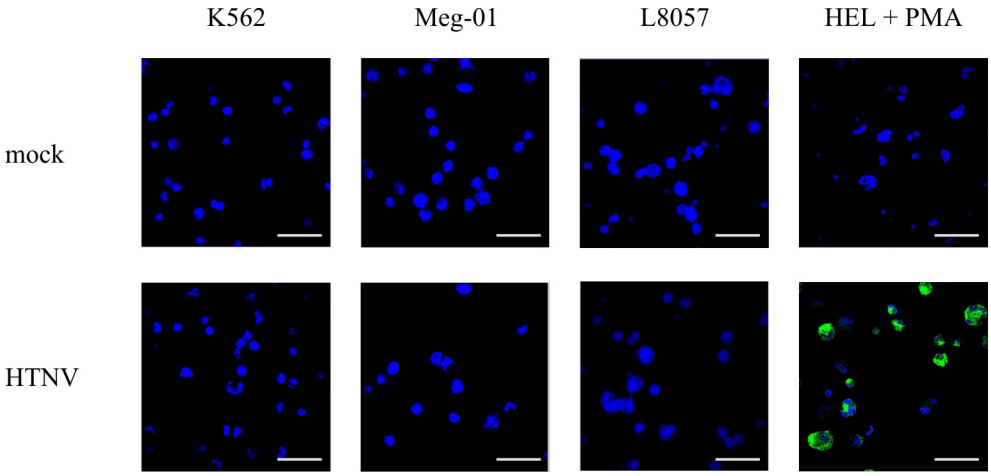

Figure 14: Analysis of K562, Meg-01 and L8057 cells for infection with HTNV.

Cells were infected with HTNV (MOI=1.5) and harvested three days pi. Cells were stained for viral N-protein using a rabbit-derived polyclonal antiserum (green). Nuclei were visualized by staining with DAPI (blue). PMA-stimulated HEL cells were used as positive control. Original magnification x60, bar = 50 µm.

In neither of the human cell lines K562 and Meg-01, nor the murine cell line L8057 viral N-protein was detected. Moreover, titration of supernatants of all cell lines also did not reveal the presence of HTNV virions (data not shown). Collectively, these results indicate that K562 cells, Meg-01 cells and the murine cell line L8057 are not susceptible to HTNV infection.

3.5 Impact of HTNV on proliferation, survival, and function of differentiating megakaryocytic cells

3.5.1 Proliferation and survival of HTNV-infected HEL cells

Next it was investigated whether HTNV infection impairs proliferation and survival of differentiating megakaryocytic cells. For this purpose, PMA-treated HEL cells were used as they represent a well-established model of megakaryocyte differentiation (Long *et al.*, 1990) and can be efficiently infected with HTNV. Counts of live unstimulated and PMA-stimulated HEL cells were determined after infection by trypan blue exclusion assay (figure 15).

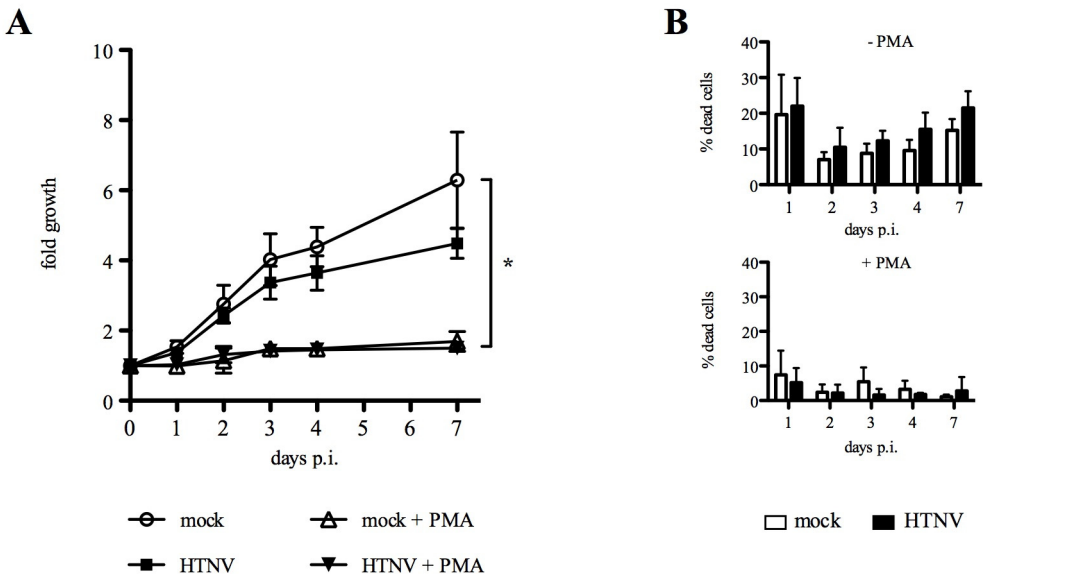

Figure 15: Proliferation and survival of mock- and HTNV-infected HEL cells.

(A) Proliferation of HEL cells. HEL cells were seeded at a concentration of 200,000 cells/ml and counted at 1, 2, 3, 4 and 7 days post infections. The growth is given on the y-axis relative to the cell number at time point 0 (fold growth). (B) Survival of HEL cells. Dead cells were visualized with trypan blue and are given as the percentage of total cells. Results shown are means ±1 SD derived from three independent experiments.

Cell growth of unstimulated HEL cells was not significantly inhibited after infection with HTNV. Stimulation of mock-infected HEL cells with PMA led to an arrest of proliferation, as described previously (Papayannopoulou *et al.*, 1983). Intriguingly, despite of PMA-induced high-level virus replication (see figure 10, p. 39) no significant differences in proliferation and survival could be detected between mock- and HTNV- infected HEL cells. In proliferating untreated cells up to 20% dead cells

accumulated, whereas less than 5% dead cells were observed in PMA-stimulated cells that did not divide. However, the percentage of dead cells between mock- and HTNV-infected HEL cells did not differ significantly. Taken together, these results demonstrate that HTNV does not alter proliferation and survival of differentiating megakaryocytic cells.

3.5.2 Analysis of programmed cell death in HTNV- and mock-infected HEL cells

Apoptosis, the programmed cell death, is part of the strongly regulated cellular metabolism. It is induced as innate response of the infected cell to eliminate the pathogen. Therefore, it was investigated whether HTNV induces apoptosis in untreated or PMA-treated HEL cells. For this purpose the TUNEL assay was used in which the enzyme terminal deoxynucleotidyl transferase labels 3'hydroxyl-DNA ends, generated during DNA fragmentation, with fluorescent dUTP. Thus, nicks in the DNA strand during apoptosis induction are visualized. HEL cells exposed to staurosporine, a microbial alkaloid that induces apoptosis in cell culture, served as a positive control (figure 16).

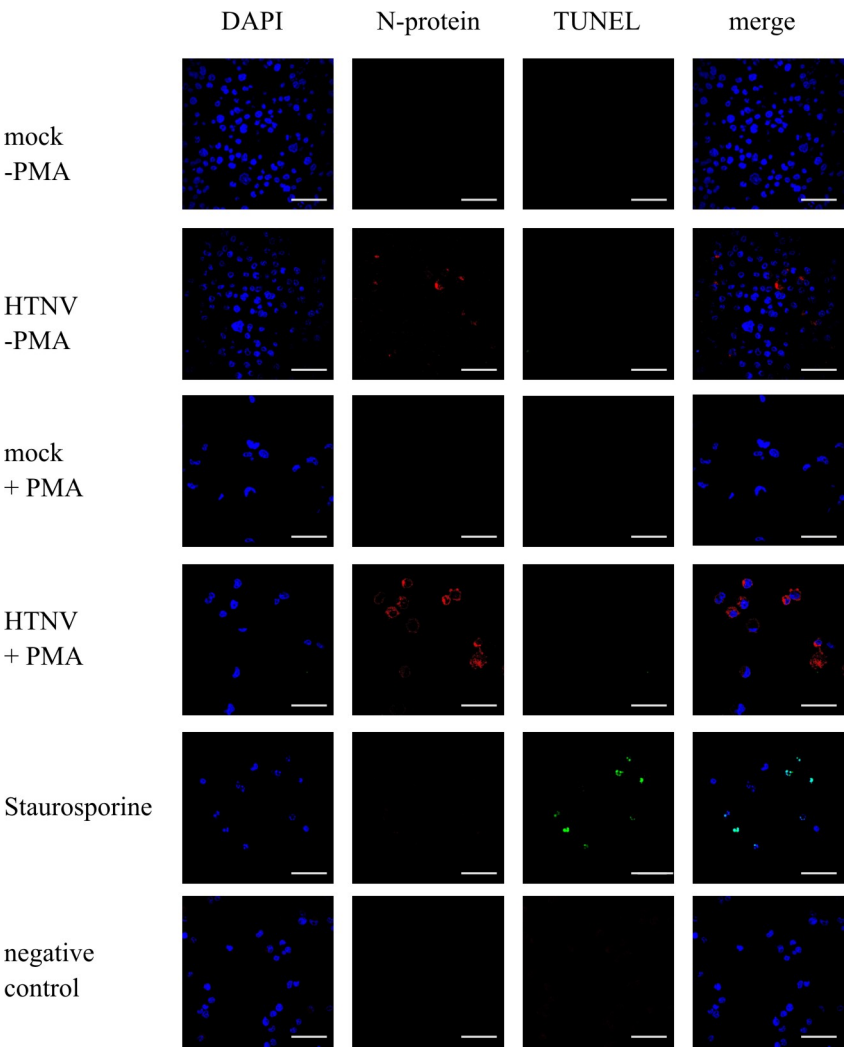

Figure 16: TUNEL assay of mock-infected and HTNV-infected HEL cells.

HEL cells were infected with HNTV (MOI 1.5) either stimulated with PMA (10 nM) (+PMA) or left unstimulated (-PMA) and analysed three days pi. As positive control HEL cells were treated with staurosporine (1µl/ml) over night. Cells were analysed for DNA degradation by TUNEL assay (green) and stained for viral N-protein using a rabbit-derived polyclonal antiserum (red). Nuclei were visualized with DAPI (blue). One representative out of three independent experiments is shown. Original magnification x60, bar = 50 µm.

DNA degradation could be observed neither in mock- nor in HTNV-infected cells. Similarly, after stimulation with PMA no DNA strand brakes were detected. Thus, even high-level replication of HTNV does not induce apoptosis in HEL cells.

To confirm the results obtained with the TUNEL assay apoptosis in HEL cells was analysed by flow cytometry. Another typical sign of apoptosis and necrosis is the loss of the asymmetric structure of the cellular membrane. Thus, phosphatidylserine, a phospholipid component usually located on the cytosolic side of the membrane can be detected on the outer leaflet of the cell membrane of apoptotic cells. AnnexinV, a

member of the annexin protein family, binds to phosphatidylserine in a calcium-dependent manner. HEL cells were infected with HTNV and treated with PMA or left untreated. Cells treated with staurosporine were used as positive control for apoptosis induction. After surface staining with AnnexinV cells were analysed by flow cytometry (figure 17).

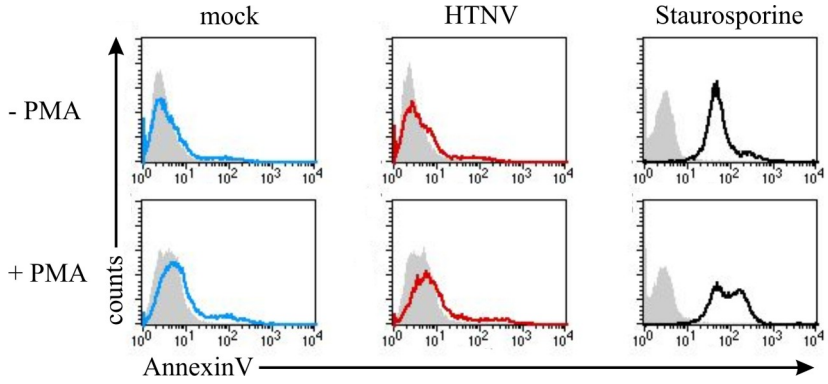

Figure 17: Flow cytometric analysis of apoptosis in mock-infected and HTNV-infected HEL cells.
Mock-infected and HTNV-infected (MOI 1.5) HEL cells were either stimulated with PMA (10 nM) (+PMA) or left unstimulated (-PMA). As a positive control HEL cells were treated with staurosporine (1µl/ml) over night. Three days pi apoptosis was detected by staining with FITC AnnexinV. Grey filled curves represent negative control (unstained cells), blue open curves indicate mock-infected cells, red open curves represent HTNV-infected cells, and black open curves indicate positive control. On the x-axis fluorescence intensity, on the y-axis cell count is given. Data shown are representatives of three independent experiments.

Only very few cells were stained with AnnexinV no matter whether mock- or HTNV-infected. Collectively these results clearly indicate that HTNV does not induce apoptosis in megakaryocytic cells.

3.5.3 Endoreplication of HTNV-infected HEL cells after PMA-induced differentiation

Differentiating megakaryocytes do not proliferate but further increase their ploidy by endoreplication (Odell & Jackson, 1968), the continued DNA synthesis without completion of mitosis and in the absence of cytokinesis. The separation process is initiated as usual: prophase, prometaphase, metaphase and anaphase I are performed. Subsequently, cells are arrested in anaphase I. As a consequence anaphase II, telophase and cytokinesis are neglected and the whole process is initiated again (Nagata *et al.*,

1997; Schulze & Shivdasani, 2004). DNA content is increased up to 64n and nuclei become multilobulated. Thus analysis of DNA content can be used to analyse the progress of megakaryopoiesis.

HEL cells undergo endoreplication after stimulation with PMA, similar to primary megakaryocytes. To test, whether infection interferes with endoreplication, analysis of DNA content was performed by staining cellular DNA with propidiumiodide. PI intercalates into nucleic acids and thus DNA content can be visualized. Results are shown in figure 18.

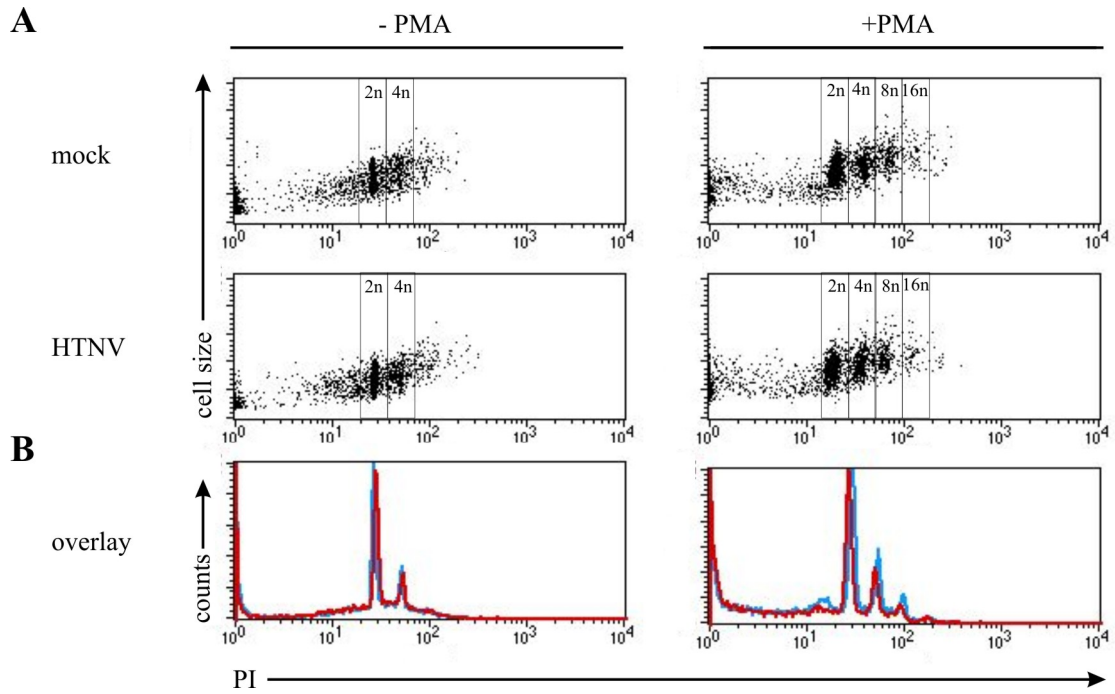

Figure 18: DNA content in mock- and HTNV-infected HEL cells 7 days pi.

(A) Dot blot analysis of DNA content of PMA stimulated (+PMA) and unstimulated (-PMA) HEL cells. On the x-axis PI fluorescence intensity, on the y-axis cell size is indicated. Gates mark different stages of DNA ploidy (n). (B) Histogram of DNA content in HEL cells. On the x-axis PI fluorescence intensity, on the y-axis relative number of cells is indicated. Mock-infected cells are shown in blue, HTNV-infected cells are shown in red. Results shown are representatives of four independent experiments.

Figure 18A shows the HEL cells in the different stages of ploidy. It can be clearly seen, that stimulation with PMA induces endoreplication and an increased ploidy. No major differences were detected between mock- and HTNV-infected cells. Table 2 demonstrates that no differences in percentages of cells in different stages are observed comparing mock- and HTNV-infected HEL cells.

Table 2: Percentage of HEL cells with different ploidy. Results shown are means derived from four independent experiments

	mock-infected -PMA	HTNV-infected -PMA	mock-infected +PMA	HTNV-infected +PMA
2n	45.5	43.5	30.5	29.75
4n	19	18	16	17
8n	-	-	6	5.75
16n	-	-	1.5	1.75

In conclusion, these findings suggest that high-level HTNV replication does not interfere with endoreplication in differentiating HEL cells.

3.5.4 Adhesive properties of HEL cells

During megakaryopoiesis direct contact between megakaryocytic cells and the extracellular matrix is of great importance for cell growth, proliferation, differentiation and maturation of the cells. Therefore, the interaction of adhesion molecules and integrins with their ligands plays a crucial role in function and development of cells (Verfaillie, 1998; Whetton & Dexter, 1993) including megakaryocytes and their progenitors. Different kinds of adhesion receptors are known, like β1, β3 or cell adhesion molecules such as Inter-Cellular Adhesion Molecule 1 (ICAM-1, CD54).

3.5.4.1 Expression of ICAM-1 on HEL cells

ICAM-1 is a transmembrane protein of the immunoglobulin superfamily expressed on a variety of cells like monocytes, epithelial cells, leukocytes and megakaryocytes (Elangbam *et al.*, 1997; Tanaka *et al.*, 1996). It is involved in inter cell adhesion, binding to leukocyte function associated antigen (LFA-1), macrophage-1 antigen (Mac-1), and fibrinogen (Diamond *et al.*, 1993; Simmons *et al.*, 1988). By binding to its ligands it triggers important immune functions: leukocyte-endothelial cell interaction and T-cell activation (Languino *et al.*, 1993). Furthermore ICAM-1 is known to interact with several pathogens like rhinoviruses or *Plasmodium falciparum* (Berendt *et al.*, 1992; Greve *et al.*, 1989). The expression of ICAM-1 on HEL cells, after infection with

HTNV, was measured to investigate a possible influence of HTNV on adhesive properties of HEL cells (figure 19).

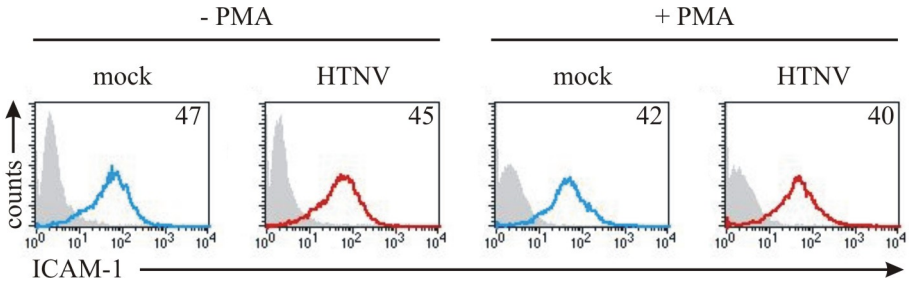

Figure 19: Expression of ICAM-1 on HEL cells.

Stimulated (+PMA) and unstimulated (-PMA) HEL cells were analysed by flow cytometry for ICAM-1 expression. Grey filled curves represent isotype control, blue open curves show specific staining of mock-infected cells and red open curves indicate HTNV-infected cells. Numbers in the upper right corner of the histograms represent mean fluorescence intensities. On x-axis fluorescence intensity, on y-axis cell count is indicated. Data shown are representatives of three independent experiments.

No significant differences in ICAM-1 expression between mock- and HTNV-infected HEL cells were detected by flow cytometry.

3.5.4.2 Adhesion of HTNV-infected HEL cells to extracellular ligands

We further analysed whether HTNV replication interferes with adhesion of HEL cells to immobilized extracellular matrix ligands. For this purpose, fibrinogen was used as a ligand for αIIbβ3, fibronectin as a ligand for α5β1, and vitronectin as a ligand for αvβ3 (figure 20).

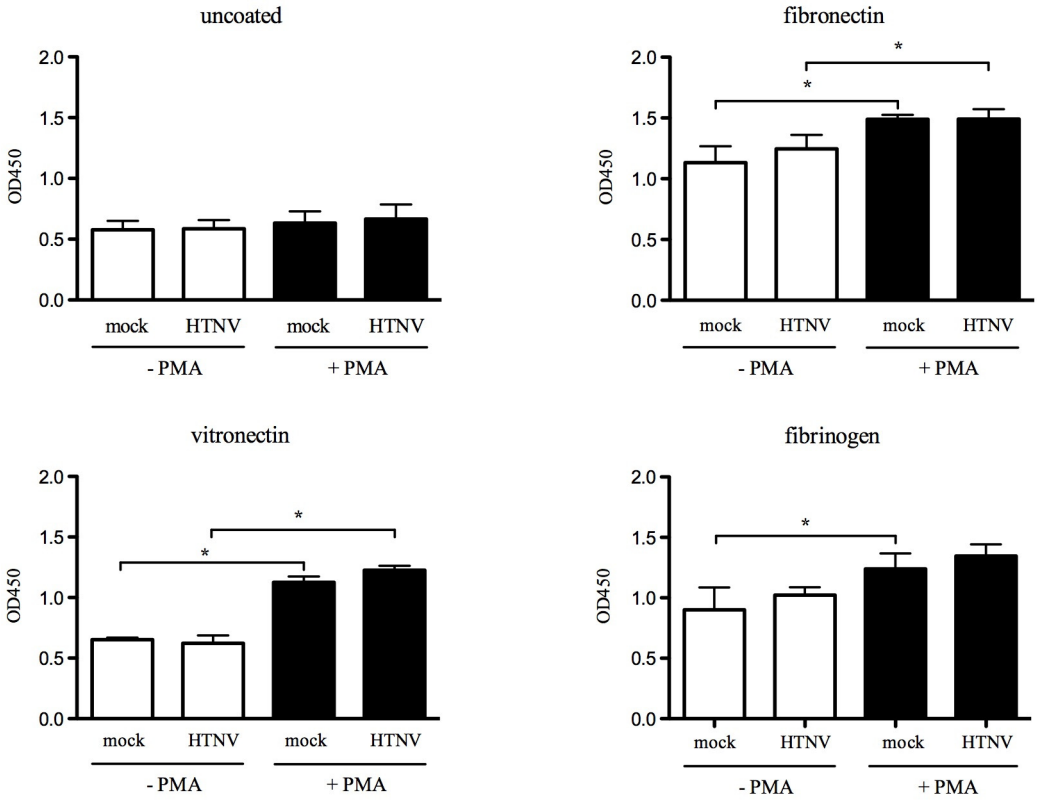

Figure 20: Adhesion of mock- and HTNV-infected HEL cells to extracellular ligands.

HEL cells were mock-infected or infected with HTNV (MOI 1.5). After three days of infection cells were allowed to adhere to extracellular ligands for 90 min. After washing off non-adhering cells, the number of cells adhering to ligands as indicated was calculated by MTT assay. Values represent means ±1 SD of triplicate determinations and are derived from three independent experiments. ∗ $p < 0,05$

After stimulation with PMA adhesion of HEL cells to fibronectin, vitronectin, and fibrinogen, was increased. Enhanced adherence after stimulation was observed with mock- and HTNV-infected cells, indicating that high-level replication of HTNV does not alter the adhesive properties of HEL cells.

3.5.5 Antiviral innate immune response in HEL cells

Any pathogen invading an organism has to cope with the innate immune system of the host. The innate receptors sensing pathogens are the pattern recognition receptors. Once pathogens are detected, an innate immune response is rapidly induced.

3.5.5.1 Expression of RIG-I in HEL cells

The RIG-I protein, a cytoplasmic PRR sensing viral RNA, is stimulated by hantaviral RNA (Lee *et al.*, unpublished data). Moreover, it was shown that the viral glycoprotein G1 (NYV) has an inhibitory effect on RIG-I signalling (Alff *et al.*, 2006; Alff *et al.*, 2008) suggesting that pathogenic hantaviruses try to subvert RIG-I function. Hence, we analysed RIG-I expression in HEL cells after infection with HTNV by immunoblot (figure 21).

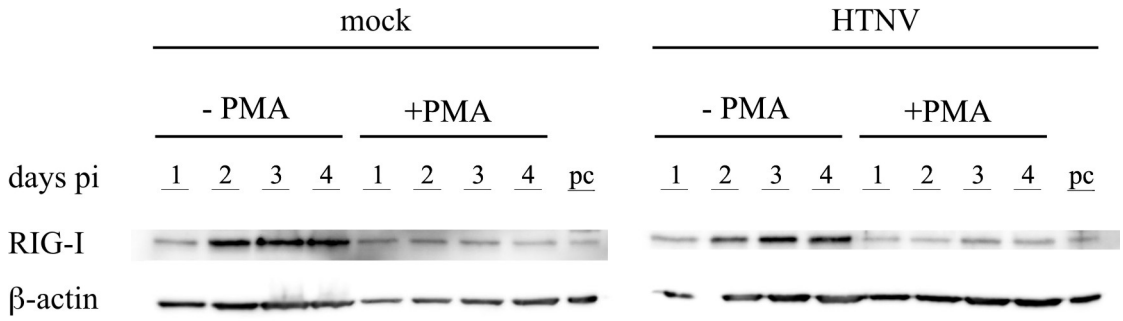

Figure 21: Expression of RIG-I in HEL cells.

Mock- and HTNV-infected HEL cells were treated with 10 nM PMA (+PMA) or left untreated (-PMA), harvested at 1-4 days pi as indicated, and analysed for RIG-I expression by immunoblot. β-actin was used as a loading control. One representative experiment out of three is shown.

HTNV infection did not influence RIG-I expression. Surprisingly, a decreased expression of RIG-I was seen after stimulation of HEL cells with PMA irrespective of whether cells were mock-infected or HTNV-infected. These data suggest that PMA interferes with RIG-I expression in megakaryocytic cells.

3.5.5.2 Expression of antiviral protein MxA in HEL cells

The antiviral protein MxA, an interferon stimulated gene, inhibits replication of hantaviruses in cell culture (Frese *et al.*, 1996; Kanerva *et al.*, 1996). In addition Kraus *et al.* showed that MxA expression is decelerated in human umbilical vein endothelial cells (HUVECs) infected with highly virulent HTNV in contrast to HUVECs infected with the rather non-pathogenic TULV (Kraus *et al.*, 2004). Therefore we decided to analyse MxA expression in HEL cells after infection with HTNV. First, we tested whether HEL cells are capable of expressing MxA and included TF-1 cells in this type of analysis. For this purpose cells were stimulated with IFN-α (figure 22).

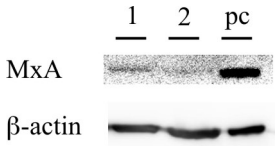

Figure 22: Expression of antiviral protein MxA in IFN-α stimulated HEL and TF-1 cells.

HEL and TF-1 cells were stimulated with IFN-α (2000U/ml) over night. Cells were harvested and analysed by immunoblot for MxA expression. As positive control Huh7 cells stimulated with IFN-α (2,000 U/ml) were used. β-actin was used as a loading control. Lane 1: IFN-α stimulated HEL cells; lane 2: IFN-α stimulated TF-1 cells; lane 3: IFN-α stimulated Huh7 cells (positive control, pc). One, out of two independent experiments is shown.

Both HEL and TF-1 cells expressed MxA, although to a much weaker extend than Huh7 cells. TF-1 cells, as they showed less IFN-α induced MxA expression than HEL cells, were excluded from further analysis. Next, HEL cells were infected with HTNV and expression of MxA was analysed by immunoblot technique (figure 23).

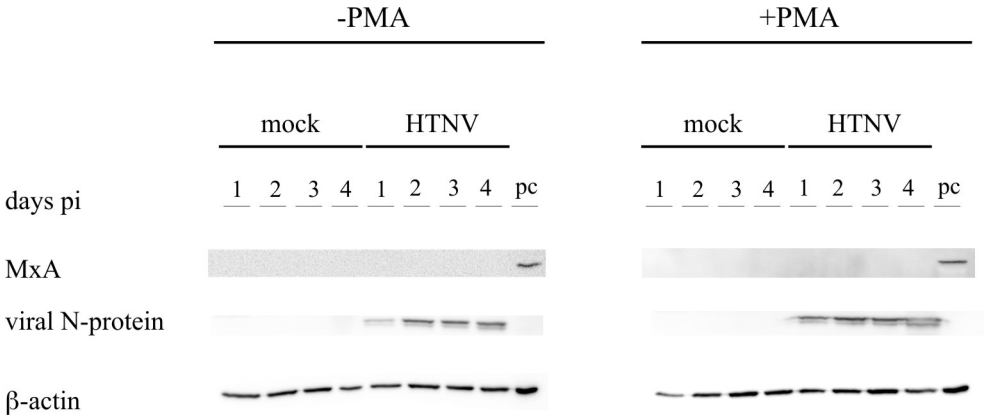

Figure 23: Expression of antiviral MxA in mock- and HTNV-infected HEL cells.

Mock-infected and HTNV-infected (MOI 1.5) HEL cells were treated with 10 nM PMA (+PMA) or left untreated (-PMA), harvested at 1-4 days pi as indicated, and analysed for expression of MxA and viral N-protein by immunoblot. As a positive control (pc) Huh7 cells, stimulated with IFN-α (2,000 U/ml) over night, were used. β-actin served as a loading control. One representative out of three independent experiments is shown.

Although capable of expressing MxA after stimulation with IFN-α, no MxA expression was detected after HTNV infection at any time point analysed. The same result was obtained with PMA-treated HEL cells despite of HTNV high-level replication in these cells. Altogether these results suggest that the antiviral MxA protein is not induced in HEL cells after infection with HTNV.

3.5.5.3 Analysis of RIG-I and MxA expression after exposure of HEL cells with TULV

So far neither viral N-protein nor virion production could be detected in TULV-infected HEL cells. Nevertheless, HEL cells expressed the β1 integrin, a cellular receptor for TULV. Thus, it is possible that a rapidly induced innate immune response prevents TULV replication, although TULV might enter HEL cells. Consequently, we analysed MxA and RIG-I expression in TULV-infected HEL cells (figure 24).

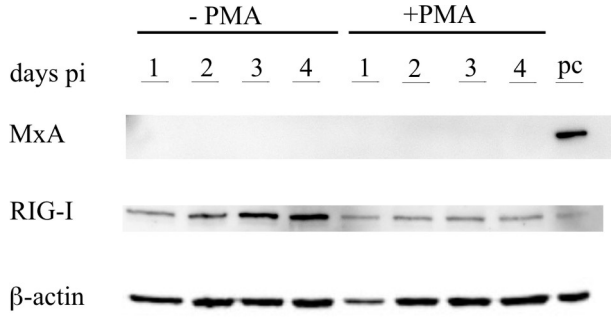

Figure 24: Analysis of MxA and RIG-I expression after exposure of HEL cells to TULV.

TULV-infected (MOI 1.5) HEL cells were treated with 10 nM PMA (+PMA) or left untreated (-PMA), harvested at 1-4 days pi as indicated, and analysed for expression of MxA and RIG-I by immunoblot. As a positive control (pc) Huh7 cells stimulated with IFN-α (2,000 U/ml) over night were used. β-actin served as a loading control. One representative out of three independent experiments is shown.

No stimulation of MxA in HEL cells, neither in unstimulated nor in PMA-stimulated, was observed after TULV infection. As described before (figure 21, p. 51) a decreased expression of RIG-I was seen after stimulation of HEL cells with PMA. Taken together, these findings indicate that, similar to HTNV, TULV does not induce the antiviral MxA protein in HEL cells.

3.5.6 Analysis of β3-integrin in HTNV-infected HEL cells

On one hand β3 is a protein that is upregulated during megakaryopoiesis and is important for megakaryocyte and platelet function, on the other hand β3 is a cellular receptor for entry of pathogenic hantaviruses. As HTNV uses β3 integrin as entry-receptor for infection, expression of this receptor might be influenced on respective cells. Altered expression of CD61 could lead to a disturbed megakaryopoiesis and thrombopoiesis. Therefore, we analysed β3 expression in HEL cells by three different

techniques: (1) surface expression by flow cytometry, (2) overall expression by immunohistochemistry and (3) overall expression by immunoblot.

3.5.6.1 Flow cytometric analysis of β3-integrin expression on HEL cells

HTNV-infected HEL cells were analysed for β3 integrin expression by flow cytometry. To clarify, if there is a general effect on megakaryopoiesis after infection, the early megakaryopoiesis marker CD41 and late megakaryopoiesis marker CD42 were analysed in parallel (figure 25).

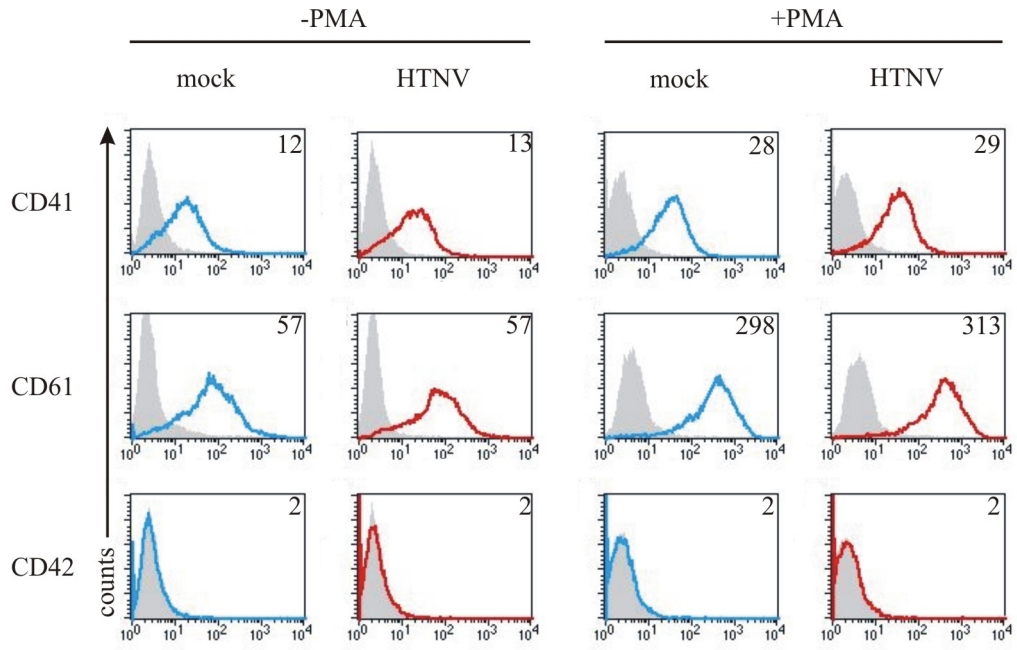

Figure 25: Expression of megakaryopoiesis markers on mock- and HTNV-infected HEL cells.
Mock-infected and HTNV-infected (MOI 1.5) HEL cells were treated with 10 nM PMA (+PMA) or left untreated (-PMA), harvested 3 days pi, and analysed by flow cytometry for expression of CD41, CD61 (β3 integrin) and CD42. Grey filled curves represent isotype control, blue open curves show specific staining of mock-infected cells and red open curves indicate HTNV-infected cells. On x-axis fluorescence intensity, on y-axis cell count is given. Numbers in the upper right corner of the histograms represent mean fluorescence intensities. Data shown are representatives of three independent experiments.

On PMA-stimulated HEL cells CD41 expression was slightly increased whereas CD61 expression was strongly upregulated as described before (figure 6, p. 34). Expression of late megakaryopoiesis marker CD42 was observed neither on unstimulated nor on PMA-stimulated HEL cells. Upregulation of CD41 and CD61 after stimulation with PMA indicates that cells differentiate into mature megakaryocytes, but

as no expression of CD42 was detected, cells are at an early differentiation state. Importantly, no significant differences in expression of CD41 and CD61 between mock- and HTNV-infected cells were detected. Collectively, these data further illustrate that HTNV does not block differentiation of megakaryocytic cells despite of high-level replication.

3.5.6.2 Immunohistochemical analysis of β3 in HEL cells

Flow cytometric analysis revealed no differences in CD61 surface expression. However, we did not gain any information about intracellular expression. For this reason we analysed β3 expression by immunohistochemistry to assess both, its extra- and intracellular expression as well as its localization. As a control, expression of CD29, the receptor for non-pathogenic hantaviruses was determined (figure 26).

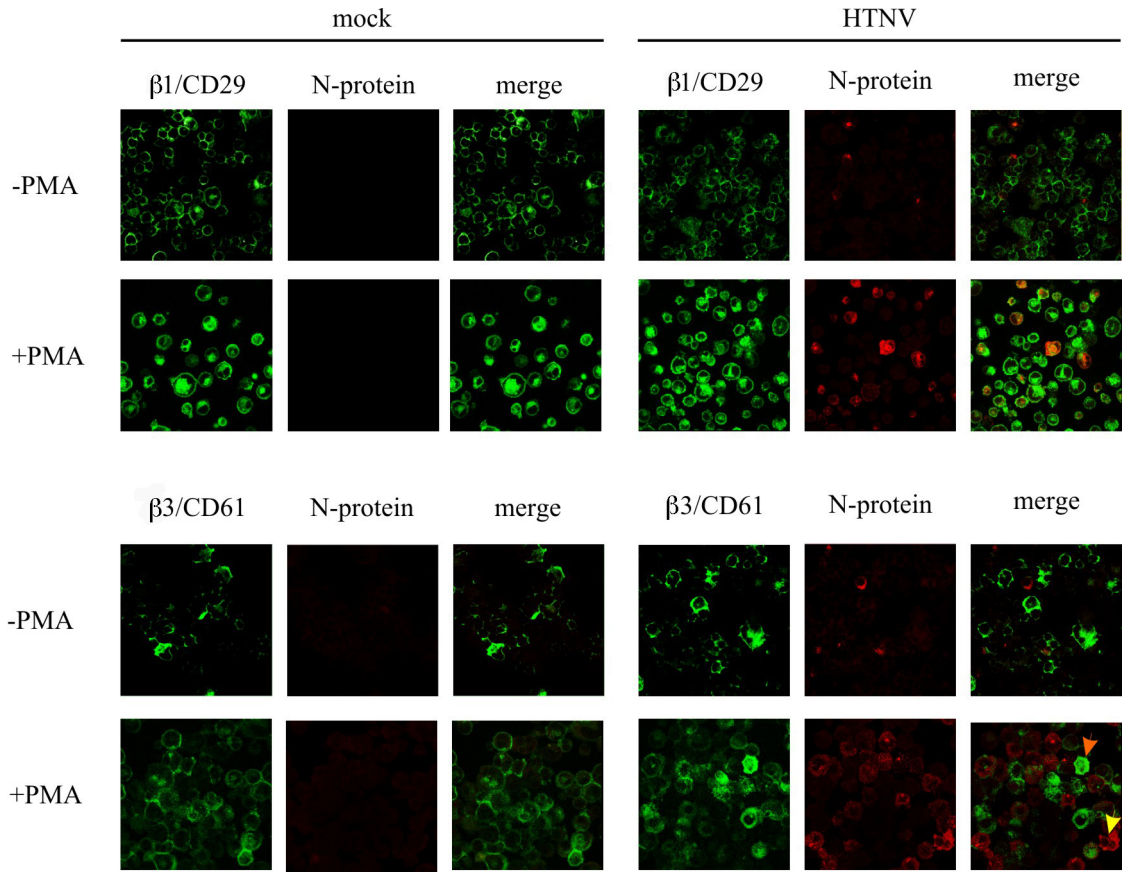

Figure 26: Expression of β1 and β3 in mock- and HTNV-infected HEL cells.

Mock-infected and HTNV-infected (MOI 1.5) HEL cells were treated with 10 nM PMA (+PMA) or left untreated (-PMA) and harvested three days pi. Cells were stained for β1 or β3 integrin (green) and viral N-protein (red). Orange arrow demonstrates cells with strong β3 expression, yellow arrow depicts cells that hardly express β3. Original magnification x60.

Both unstimulated and PMA-stimulated HEL cells showed strong β1 as well as β3 expression. No obvious differences were observed between mock- and HTNV-infected cells. Intriguingly, the merge of the staining for viral N-protein and β3 revealed that HEL cells strongly infected with HTNV hardly expressed any β3 (yellow arrow). Vice versa, in cells expressing high amounts of CD61 hardly any viral N-protein could be found (orange arrow). Whether this effect was due to downregulation or "masking" of CD61 by HTNV or whether HTNV preferably infects cells with lower CD61 expression remains to be investigated.

3.5.6.3 Immunoblot analysis of β3 in HEL cells

Finally, β3 expression of HEL cells was analysed by immunoblot to assess the total expression level of β3 integrin (figure 27).

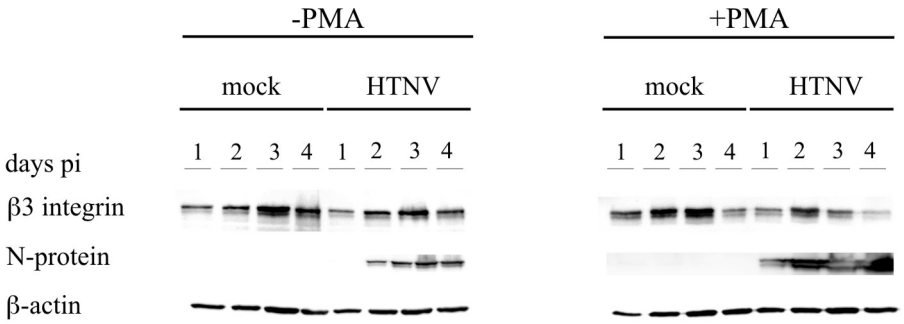

Figure 27: Analysis of β3 integrin in mock- and HTNV-infected HEL cells.

Mock-infected and HTNV-infected (MOI 1.5) HEL cells were treated with 10 nM PMA (+PMA) or left untreated (-PMA), harvested at 1-4 days pi as indicated, and analysed for expression of β3 integrin and viral N-protein by immunoblot. β-actin was used as loading control. One representative out of three independent experiments is shown.

Analysis of β3 by immunoblot revealed a slightly decreased expression of β3 in HTNV-infected HEL cells after stimulation with PMA. At day 3 and 4 after infection expression is lower in HTNV-infected, PMA-stimulated cells as compared to mock-infected, PMA-stimulated cells. Taken together, these results could imply that high-level HTNV replication downregulates intracellular β3 integrin late during infection of PMA-treated HEL cells.

3.5.7 Internalisation of integrin β1 and β3 by HEL cells

β1 and β3 integrins are not only receptors for hantaviruses, they are also important for many cellular processes like adhesion, migration, proliferation, apoptosis and phagocytosis (Caswell & Norman, 2006). Integrin-dependent migration and focal adhesion is a function of integrin recycling (Bretscher, 1992; Caswell & Norman, 2008; White *et al.*, 2007). As integrins play a key role in megakaryocyte differentiation and migration we decided to analyse trafficking of β1 and β3 integrin in HTNV-infected HEL cells by internalisation assay. Results for both integrins are shown in figure 28.

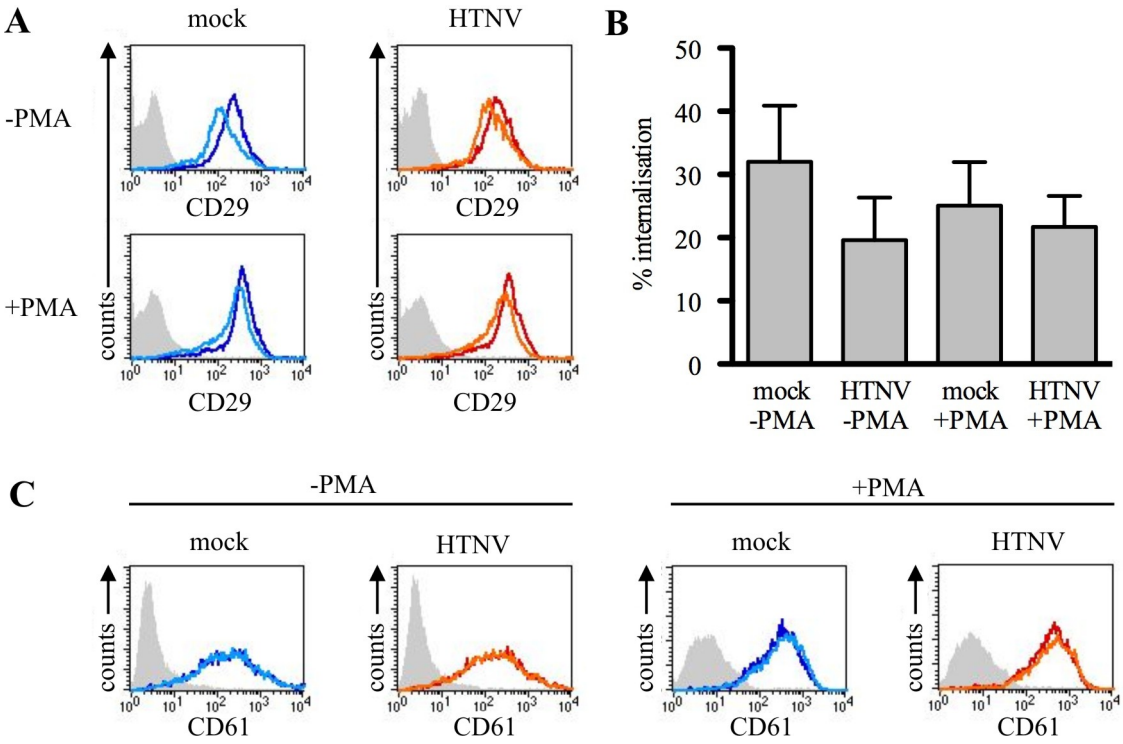

Figure 28: Internalisation assay of CD29 and CD61 on HEL cells.

Mock-infected and HTNV-infected (MOI 1.5) HEL cells were treated with 10 nM PMA (+PMA) or left untreated (-PMA). At 3 days pi cells were stained for CD29 (β1 integrin) or CD61 (β3 integrin) in culture medium for 1 h. After being washed twice, cells were incubated for 2 h at either 4°C (dark blue and dark red open curves) or 37°C (light blue and light red open curves). Subsequently, cells were stained for remaining cell surface-bound antibody with a secondary antibody. The reduction in specific staining at 37°C compared to the specific staining at 4°C represents the level of internalisation. (A) Internalisation of CD29 (β1 integrin). On x-axis fluorescence intensity, on y-axis cell count is given. One representative experiment out of three is shown. (B) Statistical analysis of the percentage of CD29 (β1 integrin) internalisation. Values represent means ±1 SD and are derived from three independent experiments. (C) Internalisation of CD61 by HEL cells. On x-axis fluorescence intensity, on y-axis cell count is given. One representative experiment out of three is shown.

For unstimulated as well as for PMA-stimulated HEL cells a slight CD29 (β1 integrin) internalisation (20-30%) was observed. But no significant differences between mock- and HTNV-infected cells could be shown as indicated in figure 28B. For β3 no internalisation could be detected. These data imply that HTNV does not interfere with trafficking of β1 integrin in HEL cells. Furthermore, expression of β3 integrin on HEL cells seems to be very stable as no internalisation was seen.

3.5.8 Organization of filamentous actin in HEL cells

The cytoskeleton is a cellular structure, consisting of several proteins and is important for cell shape, motility, transport, signal transduction and cell division. One of these proteins is actin, a monomeric protein. Actin monomers are composed as filaments, thereby forming the dynamic filamentous actin (F-actin). In megakaryocytes an intact cytoskeleton is important for the integrity of the cell and for the production of platelets (Baatout, 1996). For platelet formation an actin-dependent bending and bifurcation of the megakaryocyte is required (Italiano *et al.*, 2007). Hence disturbance of actin organization after infection with HTNV could influence platelet formation and result in abnormal platelets. We analysed structure of F-actin by staining HEL cells with rhodamine labelled phalloidine and analysed by confocal laserscan microscopy. Phalloidine is a toxin isolated from the death cap and is known to bind to F-actin. Results are shown in figure 29.

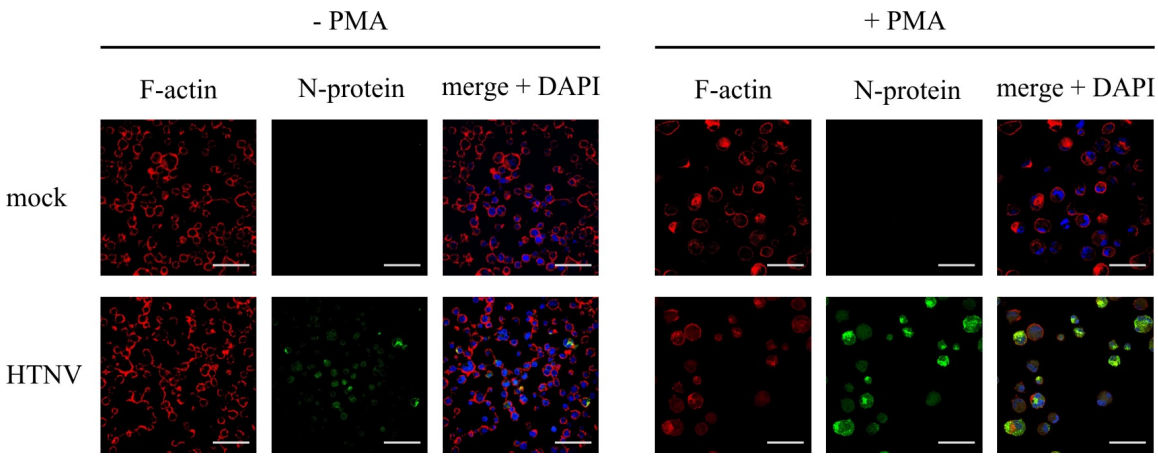

Figure 29: Immunofluorescence staining of filamentous actin in HEL cells.

Mock-infected and HTNV-infected (MOI 1.5) HEL cells were treated with 10 nM PMA (+PMA) or left untreated (-PMA). At three days pi HEL cells were stained with rhodamine labelled phalloidine to detect F-actin structures (red), viral N-protein was visualized using a rabbit-derived polyclonal antiserum (green), and DAPI to stain cellular nuclei (blue). Original magnification x60, bar = 50 µm.

Staining with phalloidine showed an increase of F-actin structures after stimulation of HEL cells with PMA. However, neither for unstimulated nor for PMA-stimulated cells differences between mock- and HTNV-infected cells were observed. In addition no colocalisation of viral N-protein with actin structures, as proved for Black Creek Canal virus, a new world hantavirus, was found (Ravkov *et al.*, 1998). In conclusion, these findings indicate that despite of high-level replication HTNV does not impair the cytoskeleton of differentiating megakaryocytic cells.

3.6 Interaction of HTNV with human platelets

Despite differences in their clinical appearance all hantavirus-associated syndromes have in common an increased microvascular permeability as well as platelet dysfunction and a dramatic decrease in platelet count (acute thrombocytopenia), which marks the beginning of vascular leakage and the hypotensive phase (Schonrich *et al.*, 2008). Platelets are anuclear cellular fragments that arise from megakaryocytes by cytoplasmic fragmentation. After release of the proplatelet into the blood biogenesis of platelets is continued in peripheral blood, mainly in the capillary bed of the lung (Behnke & Forer, 1998; Junt *et al.*, 2007), the first site of infection with HTNV. After release of platelets, cells circulate in the blood 7-10 days.

In general acute thrombocytopenia can be triggered by two mechanisms: (1) decreased production of platelets due to abnormal megakaryopoiesis/thrombopoiesis and (2) degradation of platelets in peripheral blood. Although we observed high-level replication of pathogenic HTNV in differentiating HEL cells, no direct virus-associated effect on phenotype and function could be detected in this well-established model system of megakaryopoiesis. Therefore, we decided to analyse the interaction of HTNV with platelets.

First it was analysed whether platelets can be infected with HTNV. Platelets were isolated from healthy donors and analysed for infection 24 h pi by immunohistochemistry. However, viral proteins were not detectable in human platelets by immunohistochemistry and supernatants of "infected" platelets did not contain virions (data not shown).

3.6.1 Analysis of HTNV-induced platelet activation

Although viral replication could not be demonstrated, it was nevertheless possible that HTNV interferes with platelet function. Resting platelets, circulating in the bloodstream, become activated after damage of the endothelial barrier. Several receptors are upregulated, platelets adhere and aggregate, and lesions are sealed. Activation marker CD62P (P-selectin) is stored in α-granules and transported to the cell surface after activation and several plasma proteins (e.g. platelet factor 4) are released (Kaplan *et al.*, 1979). We analysed platelet activation to verify whether hantaviruses bind to β3 integrin or further receptors and thus induce uncontrolled activation resulting in platelet exhaustion. For this purpose, platelets were isolated from healthy donors and analysed for activation by flow cytometry. Platelets stimulated with thrombin were used as a positive control. The results are shown in figure 30.

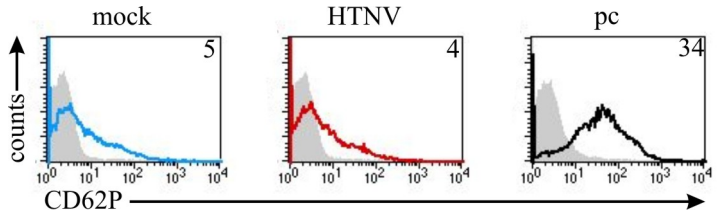

Figure 30: Flow cytometric analysis of platelets for activation marker CD62P.

Freshly isolated platelets were exposed to HTNV at MOI 0.1 and analysed for CD62P expression by flow cytometry 24 h pi. Grey filled curves indicate isotype control, the blue open curve represents mock-infected cells, the red open curve shows HTNV-exposed platelets and the black open curve shows platelets stimulated with thrombin (pc = positive control). On x-axis fluorescence intensity, on y-axis cell count is given. Mean fluorescent intensities are shown in the upper right corner of each histogram. One representative experiment out of three is shown.

No activation of platelets after exposure to HTNV was observed. The slight activation on mock-infected and HTNV-exposed platelets is probably caused by the isolation procedure. Collectively, these experiments indicate that HTNV does not trigger uncontrolled activation, which could result in "exhaustion" of platelets.

3.6.2 Interference of HTNV with essential adhesion molecules on platelets

Next to activation markers integrins αIIbβ3 (CD41/CD61), αvβ3 (CD51/CD61) and CD42 (complex of CD42 a, b, c, d) are important for platelet aggregation and attachment to diverse matrix proteins. αIIbβ3 binds to various RGD sequence containing peptides e.g. fibrinogen, fibronectin, van Willebrand factor and thrombospondin. Similarly, αvβ3, also called vitronectin receptor binds to RGD binding peptides like vitronectin and van Willebrand factor. The CD42 complex is a receptor for van Willebrand factor and thrombin. Platelets were isolated from healthy donors, "exposed" to HTNV and analysed by flow cytometry for CD61, CD41 and CD42 (figure 31).

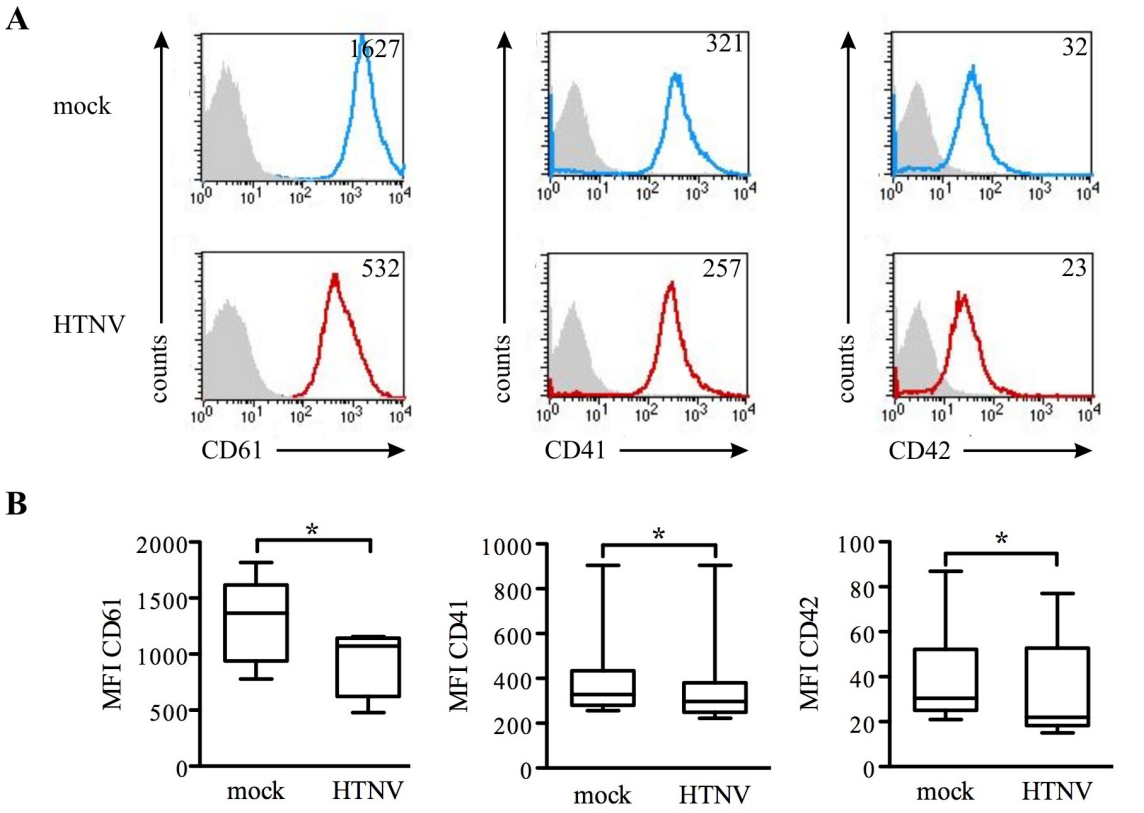

Figure 31: Expression of CD61 on mock-infected platelets and platelets exposed to HTNV.

Freshly isolated platelets were "infected" with HTNV at MOI 0.1 and analysed for expression of CD61, CD41 and CD42 by flow cytometry 24 h pi. (A) Histograms of flow cytometry analysis. Grey filled curves indicate isotype control, the blue open curve shows mock-infected cells, the red open curve represents platelets exposed to HTNV. Mean fluorescent intensities are shown in the upper right corner of histograms. One representative experiment out of 8 is shown. (B) Box-Whisker plots of mean fluorescence intensity (MFI) of CD61, CD41 and CD42 surface expression on mock-infected and HTNV-exposed platelets. CD61: n=8, CD41: n=7, CD41: n=7, $* = p < 0.05$.

Analysis of CD61, CD41 and CD42 showed a significant downregulation on the surface of platelets 24 h after exposure to HTNV. Strongest decrease in expression was observed for CD61. Receptors analysed are important for adhesion and aggregation of platelets after injury of the endothelial barrier. Collectively, these results suggest that HTNV may interfere with phenotype and function of platelets.

3.7 Activation of human lymphocytes after exposure to HTNV

After hantavirus infection in humans a vigorous antiviral T-cell response is induced. CD8+ virus-specific cytotoxic T-cells directed against viral N-protein are generated (Van Epps *et al.*, 2002) and the CD8/CD4 T-cell ratio is inverted (Huang *et al.*, 1994).

Severity of disease seems to correlate with the frequency of virus-specific CTLs, as it was shown that patients suffering from severe symptoms after SNV infection had higher frequencies of SNV-specific T-cells than patients with less severe symptoms (Kilpatrick *et al.*, 2004). After hantavirus infection long-term T-cell memory develops, although there is no sign of virus persistence (Van Epps *et al.*, 2002). To further analyse the role of lymphocytes in hantavirus infection PBMCs from buffy coats were isolated, infected with HTNV and analysed for activation marker CD69. Results are shown in figure 32.

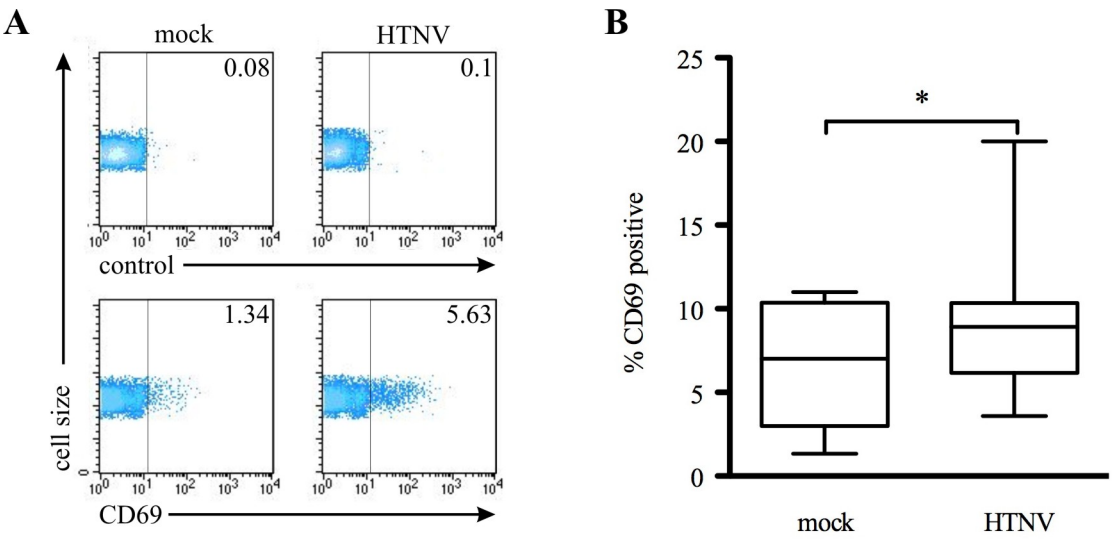

Figure 32: Analysis of lymphocytes for activation marker CD69 after infection with HTNV.
PBMCs isolated from blood of healthy donors were infected with HTNV (MOI 1.0) and analysed for expression of CD69 at three days pi by flow cytometry. (A) Dot plots of flow cytometry analysis. The y-axis indicates cell size, the x-axis shows expression of CD69. In the upper right corner the percentages of CD69-positive cells are given. One representative experiment out of seven is shown. (B) Box-Whisker plot of statistical analysis of CD69 expression on mock- and HTNV-infected lymphocytes. ∗ = $p<0.05$, n = 7.

The activation marker CD69 was upregulated on a significant proportion of lymphocytes after HTNV infection. In conclusion, these findings suggest that HTNV can activate lymphocytes derived from healthy donors *in vitro* efficiently.

3.8 HTNV-induced changes in survival, phenotype, and function of human monocytes

3.8.1 HTNV-induced survival of monocytes

Some subpopulations of monocytes, the precursor cells of both macrophages and myeloid dendritic cells, are known to be susceptible to hantaviruses (Markotic *et al.*, 2007). Next to its role as precursor cells, monocytes are important immune cells. They phagocytose pathogens and are involved in antigen presentation. We wanted to test the effect of HTNV on monocytes. Therefore, monocytes isolated from buffy coats that had been derived from healthy donors were infected with HTNV and analysed by flow cytometry for apoptosis (figure 33).

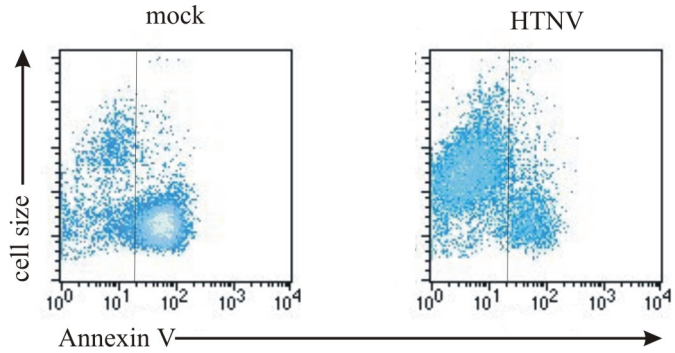

Figure 33: Analysis of apoptosis in mock- and HTNV-infected monocytes.

Monocytes were isolated from peripheral blood mononuclear cells derived from healthy human donors by using the Monocytes Isolation KitII from Miltenyi Biotec. Mock- and HTNV- infected (MOI 1.0) monocytes were analysed for apoptosis by AnnexinV-staining three days pi. The x-axis depicts AnnexinV-staining, the y-axis shows cell size of monocytes. One, out of two independent experiments is shown.

As described previously mock-infected monocytes underwent apoptosis after isolation and culturing for some days (Keisari, 1996). Accordingly, these cells showed the morphology of dying cells, when analysed by light microscopy (data not shown). In striking contrast, HTNV-infected monocytes survived and only a low percentage of cells could be stained with AnnexinV. Additionally, the HTNV-cells had the morphology of live cells. In conclusion, HTNV infection enables monocytes to survive in cell culture.

3.8.2 HTNV-driven differentiation of monocytes

Having established that HTNV-infection allows human monocytes to survive they were analysed by flow cytometry for expression of typical markers of monocytes and dendritic cells. However, as monocytes die off soon after a short period of culturing mock-infected monocytes were analysed right after isolation for comparison. The following proteins were analysed as marker molecules: CD14, a GPI-anchored 356 aa glycoprotein expressed on monocytes and macrophages; CD83, a single-chain type-1 glycoprotein expressed on mature myeloid DCs; CD86, a protein belonging to the immunoglobulin family, constitutively expressed on DCs and in low levels on monocytes; and DC-SIGN, a 404 aa glycoprotein that is predominantly expressed on dendritic cells. Results are shown in figure 34.

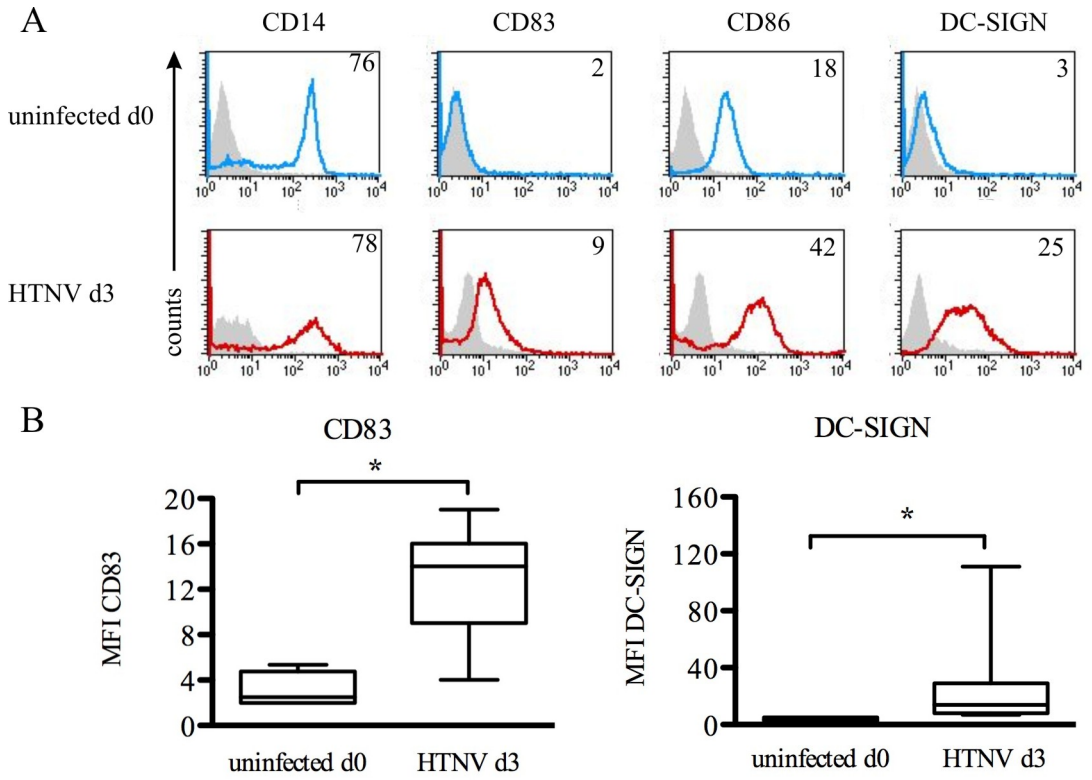

Figure 34: Flow cytometric analysis of uninfected and HTNV-infected monocytes.

(A) Monocytes were infected with HTNV (MOI 1) and analysed three days pi by flow cytometry for CD14, CD83, CD86 and DC-SIGN (red open curves). For comparison surface expression of uninfected monocytes (d0) is shown (blue open curves). Grey filled curves represent isotype controls. The x-axis shows staining with specific antibody, the y-axis indicates cell count. One representative experiment out of three is shown. (B) Box-Whisker plot of statistical analysis of expression of CD83 and DC-SIGN on mock-infected and HTNV-infected monocytes. ∗ = $p<0.05$, n= 3.

Flow cytometric analysis of monocytes showed that HTNV-infected monocytes lose their classical monocytic expression profile. Expression of CD14 hardly changed, but upregulation of macrophage/dendritic cell markers CD83, CD86, and DC-SIGN was detected. Upregulation of CD83 and DC-SIGN was shown to be significant. Collectively, these data demonstrate that HTNV induces differentiation of infected monocytes towards a cell type resembling macrophages/DCs.

4 Discussion

In order to understand the mechanisms of hantaviral pathogenesis, until now research was focused on mechanisms which directly damage the endothelial barrier resulting in haemorrhages. However, in a healthy person microvascular lesions occur daily, but remain undetected as long as enough and fully functional platelets are available. Thus bleedings are prevented. As soon as platelets are defect and/or platelet count is too low, lesions cannot be sealed anymore and haemorrhages may appear. During hantavirus infection not only damage of the endothelial barrier but reduced platelet count and platelet dysfunction play a pivotal role (Cosgriff, 1991b). This suggests an involvement of cells of the haemostatic system in hantavirus pathogenesis. In this thesis the hantavirus-induced mechanisms leading to acute thrombocytopenia and platelet dysfunction were analysed. Moreover, the interactions of hantaviruses with cells of the human immune system were investigated as there is evidence that the immune system itself contributes to hantavirus-associated pathogenesis (immunopathogenesis).

4.1 Megakaryocytic cells as a tool for studying megakaryopoiesis

Megakaryopoiesis is the development of a haematopoietic stem cell into mature megakaryocytes. This process takes place via several steps of maturation and differentiation in the bone marrow. The percentage of megakaryocytes ranges from 0.03% to 0.06% of all nucleated cells in the bone marrow (Saito, 1997), hence isolation of a large number for biochemical analysis is hardly possible. Furthermore *ex vivo* differentiation of CD34+ cells, isolated from umbilical cord blood, is very complex and difficult. Cells require special cytokine cocktails (e.g. TPO, stem cell factor, interleukin (IL) 1) to survive and developing megakaryocytes react sensitively to any change of condition (e.g. temperature, medium concentration) (Maurer *et al.*, 2000). Thus working with primary megakaryocytes is complex, expensive and the yield of cells is usually very low.

Therefore megakaryocytic cell lines, established from blood or bone marrow of patients with leukemia represent a precious alternative for analysing features of megakaryocytic cells. Cells are easy to maintain in culture and can be expanded to the desired number. They have no or little variation and are not as cost-intensive as primary cells. However one has to keep in mind that many megakaryocytic cell lines share

multilineage features, holding characteristics of erythroid, myeloid and megakaryocytic cells. Nevertheless these cell lines represent an excellent tool for studying megakaryopoiesis (Saito, 1997).

4.2 Hantaviruses and megakaryocytic cells

4.2.1 Susceptibility of megakaryocytic cells to hantavirus infection

In this thesis, established megakaryocytic cell lines were tested whether they can be infected with hantaviruses. For the first time, we showed that pathogenic but not apathogenic hantaviruses target megakaryocytic cells. HTNV infected TF-1 and HEL cells in contrast to apathogenic PHV and TULV. Further human megakaryocytic cell lines (K562, Meg-01) and a murine megakaryocytic cell line (L8057) could not be infected with any hantavirus tested. TF-1 cells represent very undifferentiated cells (Kitamura *et al.*, 1989). K562 and HEL cells are early megakaryoblast-like cells (Gewirtz *et al.*, 1982; Papayannopoulou *et al.*, 1988; Papayannopoulou *et al.*, 1983; Saito, 1997), whereas HEL cells express more typical megakaryocytic markers. K562 cells only commit towards the megakaryocytic lineage after stimulation with PMA and hold less megakaryocytic characteristics than HEL cells. Meg-01 cells represent a later stage, approximately the late megakaryoblast (Ogura *et al.*, 1985; Takeuchi *et al.*, 1991). Altogether, it seems that HTNV only infects cells of the early stage of megakaryopoiesis.

Infection of megakaryocytic cells in the bone marrow of humans could influence megakaryopoiesis at different stages. This could result in defective megakaryocytes, less platelet production or “abnormal” platelets that have lost their functions. Several viruses are known to infect megakaryocytes and thus cause decreased platelet formation, e.g. human immunodeficiency virus (HIV) (Scaradavou, 2002; Zucker-Franklin & Cao, 1989) and dengue virus (Basu *et al.*, 2008). For HIV it is known that both reduced platelet production due to bone marrow suppression and the production of anti-platelet antibodies in peripheral blood contribute to virus-associated thrombocytopenia (Scaradavou, 2002). Thrombocytopenia is an important event in hantavirus-induced pathogenesis. Reduced platelet count may develop by decreased formation of platelets and/or increased elimination of platelets. Thus interaction of

hantaviruses with megakaryocytes and/or platelets could explain hantavirus-associated thrombocytopenia.

4.2.2 Expression of hantavirus receptors on megakaryocytic cells

The megakaryocytic cell lines TF-1, HEL, K562 and Meg-01 cells express β1-integrin (CD29), β3-integrin (CD61) and CD55/DAF, which facilitate hantavirus entry (Gavrilovskaya *et al.*, 1999; Gavrilovskaya *et al.*, 1998; Krautkramer & Zeier, 2008). However, we could hardly detect surface expression of gC1qR, which has been reported to enhance entry of HTNV infection into human lung epithelial A549 cells (Choi *et al.*, 2008), on any cell line analysed. Thus, except of unstimulated K562 cells, which do not express surface β3, all cell lines analysed were equipped with canonical hantavirus receptors, but only TF-1 and HEL cells were infectable. This observation suggests that besides the known surface receptors, hantavirus infection is dependent on additional factors. Possibly a hitherto unknown coreceptor is required for infection with apathogenic and/or pathogenic hantaviruses that is missing on some of the cells analysed. Alternatively, as integrins exist in various splice variants, it is possible that hantaviruses only use one special variant of the integrin, not expressed on all cell lines tested in this thesis (Fornaro & Languino, 1997; Svineng *et al.*, 1998). HTNV only enters the cell when β3 is expressed in its inactive (bent) conformation (Raymond *et al.*, 2005). Thus, infection can only happen when β3 is not activated. Moreover, it is possible that viral particles enter the cells but do not replicate because the cellular milieu lacks certain proteins that are necessary to start the replication process. Finally, it has to be considered that a cell autonomous defence mechanism actively blocks viral replication.

In summary, productive infection relies on several conditions that might not be given in all megakaryocytic cell lines tested and the fact that highly pathogenic HTNV but not less pathogenic TULV and PHV could establish infection in megakaryocytic cells, points towards an important role of this cell type in HFRS and possibly HCPS.

4.2.3 Switch to High level hantavirus replication after PMA treatment

Stimulation of HEL cells with PMA increased hantavirus titers up to two logs. Although hantavirus receptor (β3) surface expression is increased after stimulation, the PMA-induced upregulation does not explain increased titers, as stimulation with PMA before and after infection resulted in similar titers. It is possible that stimulated HEL cells provide better conditions for virus growth, such as increased availability of the ER-Golgi intermediate compartment, which is required for hantaviruses replication (Ramanathan *et al.*, 2007), or an enhanced nucleotide metabolism. However, PMA stimulation of HTNV-infected VeroE6 cells did not increase virus titers suggesting that PMA stimulates a pathway that is not affected or absent in these cells. For example PMA could have an inhibitory effect on induction of type I IFN or type I IFN signalling which is not obvious in VeroE6 cells that lack IFN type I genes (Diaz *et al.*, 1988). Hantaviruses trigger IFN I and thereby the innate immune response. Thus, increased viral replication due to stimulation with PMA could be caused by an indirect effect: a diminished innate immune response. This finding is supported by the fact that expression of innate cytoplasmic sensor RIG-I is decreased after stimulation with PMA (figure 21, p. 51). It was shown that RIG-I senses viral N-protein encoding hantaviral RNA (Lee *et al.*, unpublished data) and is a target of hantaviral immune evasion mechanisms (Alff *et al.*, 2006). Type I IFN is triggered through RIG-I signalling, which binds to type I IFN receptor. Subsequently the receptor-associated tyrosine kinases janus protein tyrosine kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) are activated resulting in posphorylation of signal transducer of activation and transcription (STAT1) and STAT2. These dimerize and translocate into the nucleus inducing transcription of multifarious genes, which subsequently induce transcription of a multitude of IFN-stimulated genes encoding antiviral effector components (Sadler & Williams, 2008) e.g. double-stranded RNA-dependent protein kinase (PKR) (Stark *et al.*, 1998).

Previously PMA has been shown to target components of the innate immune system. Inhibition of IFN-α stimulated tyrosine phosphorylation of transcription factors STAT1α, STAT2, STAT3 and TYK2 was shown after treatment of primary monocytes with PMA. Hence, PMA stimulation of monocytes resulted in inhibition of JAK/STAT pathway (Petricoin *et al.*, 1996). Furthermore, PMA is known to activate the RAS/MAPK (mitogen-activated protein kinases) pathway (Marquardt *et al.*, 1994), which controls many biological processes. For example, activated RAS inactivates

PKR, an important player of the antiviral innate immune response (Mundschau & Faller, 1992). For vesicular stomatitis virus (VSV) (Battcock *et al.*, 2006), reovirus (ReoV) (Strong *et al.*, 1998), herpes simplex virus type 1 (HSV1) (Farassati *et al.*, 2001) and ebola virus (EBOV) (Strong *et al.*, 2008) it was demonstrated that virus growth is enhanced in RAS-transformed fibroblasts, showing that downregulation of antiviral innate immune responses induced by type I IFN increases viral replication. Altogether, PMA could enhance replication of hantaviruses through modulation of several key pathways including the RIG-I pathway, the JAK/STAT pathway and the RAS/MAPK pathway, which are shown in figure 35.

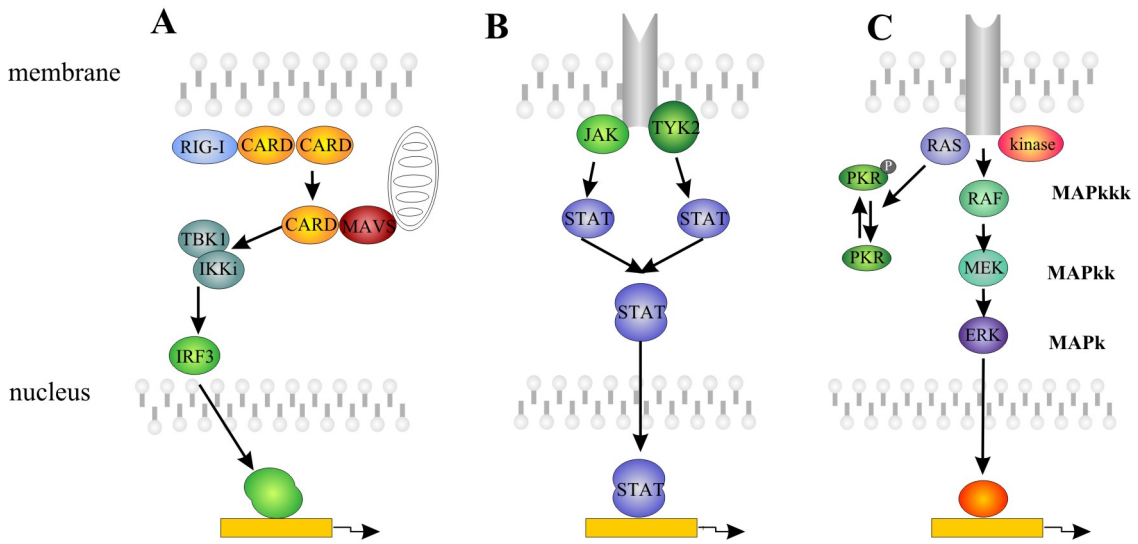

Figure 35: Simplified schemes of cellular pathways influenced after PMA stimulation.
(A) RIG-I pathway, (B) JAK/STAT pathway and (C) RAS/MAPK pathway

Intriguingly, the PMA-associated effects could also be relevant *in vivo* as thrombopoietin, the central regulator for megakaryopoiesis *in vivo,* activates RAS/MAPK signalling (Kirito & Kaushansky, 2006). Thus, TPO-induced megakaryocyte differentiation *in vivo* could be linked to enhanced replication of hantaviruses in megakaryocytic cells (figure 37, p. 77).

4.2.4 Unaltered phenotypic and functional differentiation of HTNV-infected megakaryocytic cells despite high-level HTNV replication

PMA-treated HEL cells represent a well-established model system of megakaryocyte differentiation. After PMA-treatment HEL cells stop proliferating and further differentiate into cells resembling more mature megakaryocytes, indicated by an increase in cytoplasm, ploidy and upregulation of megakaryocyte/platelet proteins (Long *et al.*, 1990). Therefore, we analysed in detail whether the switch to high-level HTNV replication in HEL cells interfered with their differentiation. Only a slight but not significant reduction in proliferation of unstimulated HTNV-infected HEL cells was observed. Importantly, independent of whether HEL cells were HTNV-infected or mock-infected, PMA stimulation yielded in arrest of proliferation, begin of differentiation and initiation of endoreplication, an important step in megakaryocyte development. In addition no signs of HTNV-induced cell death, neither by analysis of DNA strand brakes, nor by staining with AnnexinV were seen.

Adhesion of megakaryocytes to the extracellular matrix and among each other is an essential process during megakaryopoiesis. When cells were analysed for expression of adhesion molecule ICAM-I and for adhesion to components of the extracellular matrix, no differences were detected between mock and HTNV-infected cells. Integrin-dependent migration and adhesion is closely connected to integrin recycling (Caswell & Norman, 2006) and disturbance in integrin trafficking could influence megakaryocyte development and function. Therefore, internalisation capacities of β1 and β3 were analysed. However, no significant differences were found between mock and HTNV infected HEL cells. Taken together, high level replication of HTNV did not interfere with proliferation, viability, adhesion and endoreplication of megakaryocytic cells.

Megakaryocyte differentiation is accompanied by consecutive upregulation of surface expression of key markers CD41 (IIb integrin), CD61 (β3 integrin) and CD42. After HTNV infection no differences were observed in surface expression of these differentiation markers as compared to mock-infected cells. Intriguingly, immunofluorescence analysis localized HTNV preferably in HEL cells with low or no β3 expression. In contrast, cells with strong β3 expression were mostly uninfected. Thus, it was possible that HTNV downregulates β3 expression. To further examine this aspect we performed immunoblot analysis of β3 integrin 1-4 dpi. We found that overall

expression of CD61 was only slightly decreased over time in PMA-stimulated HEL cells after infection. This indicates that HTNV has only a weak influence on overall cellular β3 expression. It appears unlikely that HTNV "masks" the β3 integrin, e.g. by changing the conformation recognized by the anti-β3 antibody, as FACS analyses of HTNV-infected cell cultures using the same reagent revealed unaltered β3 surface expression. It is more likely that HTNV preferably infects cells with lower CD61 expression, which represent more early stages of megakaryocyte differentiation. Supporting this view, HTNV infects TF-1 and HEL cells, but not Meg-01 cells. Moreover, upregulating β3 on HEL cells before infection by PMA treatment did not further increase viral replication as compared to cells that were treated with PMA 1 h after infection. In conclusion, HTNV seems to target early stages of megakaryocytes without causing any alteration in their further differentiation.

4.2.5 Unaltered cytoskeleton in megakaryocytic cells after HTNV infection

For BCCV colocalization of viral N-protein and F-actin in VeroE6 cells was detected and a role of actin in viral assembley was assumed (Ravkov *et al.*, 1998). In contrast, for HTNV, it was shown that N-protein associates with microtubules, but not with actin in VeroE6 cells (Ramanathan *et al.*, 2007). After analysis of F-actin and HTNV nucleoprotein in HEL cells by immunohistochemistry no alterations in cellular F-actin structures and no strong colocalization could be shown. This finding further supports the view that HTNV does not interfere with cytoskeletal structures of megakaryocytes and, as an intact cytoskeleton is essential for megakaryopoiesis and platelet formation, with megakaryopoiesis.

4.2.6 Immunological aspects of HTNV infection of megakaryocytic cells

The hantaviral N-protein is the most abundant viral protein and a strong immune response is directed against it in humans. It induces early IgM response and is known to interact with several cellular proteins (Kaukinen *et al.*, 2005), e.g. actin filaments (Ravkov *et al.*, 1998), the death-domain associated protein Daxx, a protein that interferes with various further cellular proteins (Li *et al.*, 2002; Salomoni & Khelifi, 2006), ubiquitin-like modifier-1 (SUMO-1) (Maeda *et al.*, 2003), and antiviral MxA (Khaiboullina *et al.*, 2005). Differentiating megakaryocytes could contribute to the

induction of a strong immune response against HTNV by releasing high numbers of virions containing protein.

No expression of MxA was detected in unstimulated or PMA-stimulated HTNV-infected HEL cells. Possibly, HTNV subverted the type I IFN-induced immune response in unstimulated HEL cells. Alternatively, low-level replication in unstimulated HEL cells was not able to trigger type I IFN production. In contrast, lack of MxA expression in PMA-stimulated HEL cells might be due to general inhibition of the type I interferon pathway by PMA.

No cytopathic effect, even after stimulation of high-level replication, was found in HTNV-infected megakaryocytic cells. This suggests that thrombocytopenia occurs partly due to elimination of megakaryocytic cells by CTLs. In line with this view the density of human leukocyte antigen (HLA) class I molecules on HTNV-infected cells is upregulated (Pritesh Lalwani, personal communication) thereby most likely increasing the vulnerability to antiviral CTL attack. The aspect is supported by the fact that the frequency of virus-specific CTLs correlates with the severity of disease in HCPS patients (Kilpatrick *et al.*, 2004). This implicates that more widespread infection of megakaryocytes renders more megakaryocytes susceptible to CTL attack resulting in a more drastic drop in platelet count. Indeed, analysis of blood showed that in patients suffering from NE or HCPS high viral loads correlate with low platelet count (Kilpatrick *et al.*, 2004; Rasche *et al.*, 2004; Terajima *et al.*, 1999). Moreover, it was observed that immunosuppressive corticosteroid therapy, which suppresses CTL responses, increases platelet count in NE patients (Dunst *et al.*, 1998; Seitsonen *et al.*, 2006) and in patients suffering from less severe HFRS a high frequency of virus-specific T-cells producing IFN-γ has been observed (Wang *et al.*, 2009). IFN-γ controls viral spread through non-cytolytic clearance avoiding excessive elimination of infected cells (Guidotti & Chisari, 2001). Altogether it seems that the immune system and not inhibition of megakaryopoiesis after infection contributes to thrombocytopenia.

4.3 Interaction with platelets

Besides a dramatic decrease in platelet count at the beginning of vascular leakage and the hypotensive phase, platelet dysfunction has been reported in patients with hantavirus-associated syndromes (Cosgriff, 1991a; Cosgriff *et al.*, 1991b; Lee, 1987;

Lee *et al.*, 1983). The hantavirus receptor β3 is expressed at a high density on platelets, where it has essential functions in processes like adhesion, migration and signalling (Shattil, 1995). On unactivated, circulating platelets β3 is expressed in its bent conformation, the conformation that pathogenic hantaviruses use for cell entry (Raymond *et al.*, 2005).

We could not detect HTNV infection of platelets *in vitro*, but recently it was detected that pathogenic hantaviruses bind to quiescent platelets (Gavrilovskaya *et al.*, 2010). Susceptibility of platelets to HTNV infection is unlikely anyway as hantavirus growth requires the ER-Golgi intermediate compartment (Ramanathan *et al.*, 2007). In addition, no activation of platelets via P-selectin (CD62P) was seen after exposure of platelets to HTNV. However, surface markers CD61, CD41, and CD42 were downregulated 24 h after exposure of platelets to HTNV. This suggests that after contact of HTNV with integrin receptors on platelets signalling is induced, resulting in downregulation of adhesion markers. This implies loss of platelet function and reduced clotting capacities. Though, it is difficult to prove loss of coagulation capacities, due to experimental limitations in L3 biosafety level. Supporting our finding, Cho *et al.* found that expression of van Willebrand receptor (CD42) is reduced up to 86 % after HTNV infection of human umbilical vein endothelial cells possibly delaying coagulation (Cho *et al.*, 2007).

Patients who develop hemorrhagic fever suffer from bleedings, thrombocytopenia, coagulopathy and disseminated intravascular coagulation (Lee *et al.*, 1989; Lee *et al.*, 1983). These bleedings resemble those found in Glanzmann's disease, an inherited bleeding disorder, suggesting common causes (Gavrilovskaya *et al.*, 2008; Raymond *et al.*, 2005). On platelets of those patients αIIbβ3 is absent or dysfunctional, caused by mutations in genes of respective proteins. This leads to decreased ability of platelets to aggregate and thereby to defects in primary haemostasis (Nurden, 2006). We found decreased CD61 expression on platelets exposed to HTNV, which could substantially contribute to platelet dysfunction observed in patients with HFRS (Cosgriff, 1991a; Cosgriff *et al.*, 1991b; Lee, 1987; Lee *et al.*, 1983). In Glanzmann's thrombasthenia patients who suffer from acute bleedings are treated with platelet transfusions (Bellucci & Caen, 2002). This therapy could help to prevent strong decrease in platelet count in patients with severe symptoms after hantavirus infection (Dietl *et al.*, 2008; Kim *et al.*, 2003) and thus avert further fatal symptoms.

4.4 Interference of pathogenic Hantaviruses with the haemostatic system

HTNV infects cells that are early stages of megakaryopoiesis. Furthermore HTNV interacts with platelets without infecting these cells. This Interaction leads to decreased expression of adhesion markers CD61, CD41 and CD42 on the platelet surface.

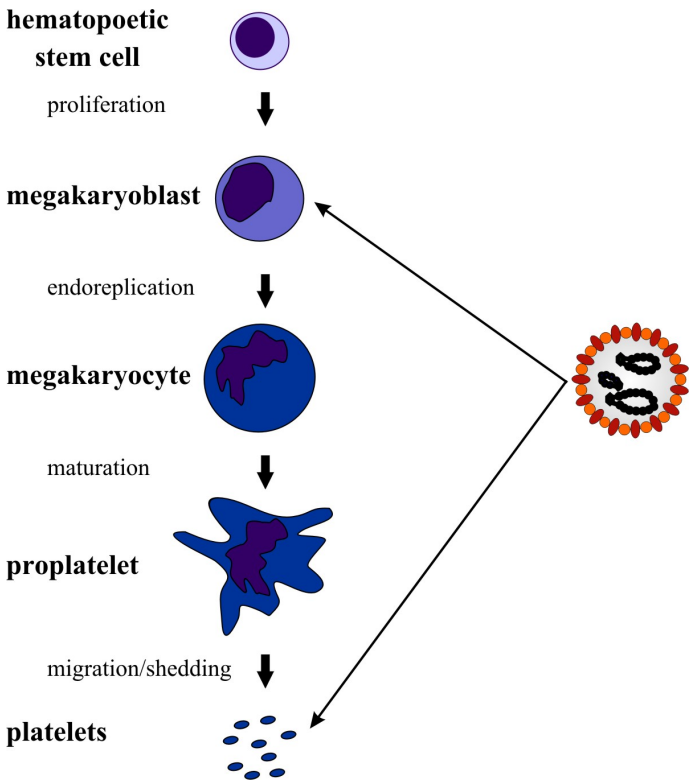

Figure 36: Cells of the haemostatic system as targets for HTNV.

Thin arrows demonstrate sites of interaction between virus and cells. Pathogenic HTNV infects the early megakaryoblast and interacts with platelets in humans.

Taken together, our data provide evidence for two different mechanisms by which pathogenic hantaviruses interfere with haemostasis and exploit megakaryopoiesis to produce virions (figure 36). Firstly, HTNV infects megakaryocytic cells that resemble early megakaryoblasts without causing any obvious cytopathic effect. Infected megakaryocytic cells present hantaviral antigens in the context of HLA class I molecules, which are likely targets for CTLs. This could lead to reduced platelet production and acute thrombocytopenia. As a compensatory response the bone marrow increases megakaryopoiesis to counteract the low platelet count. In this way the pool of megakaryocytic cells susceptible to virus infection is enlarged, further enhancing the

virus load. The process is demonstrated in figure 37. In humans the virus is finally eliminated by a strong immune response.

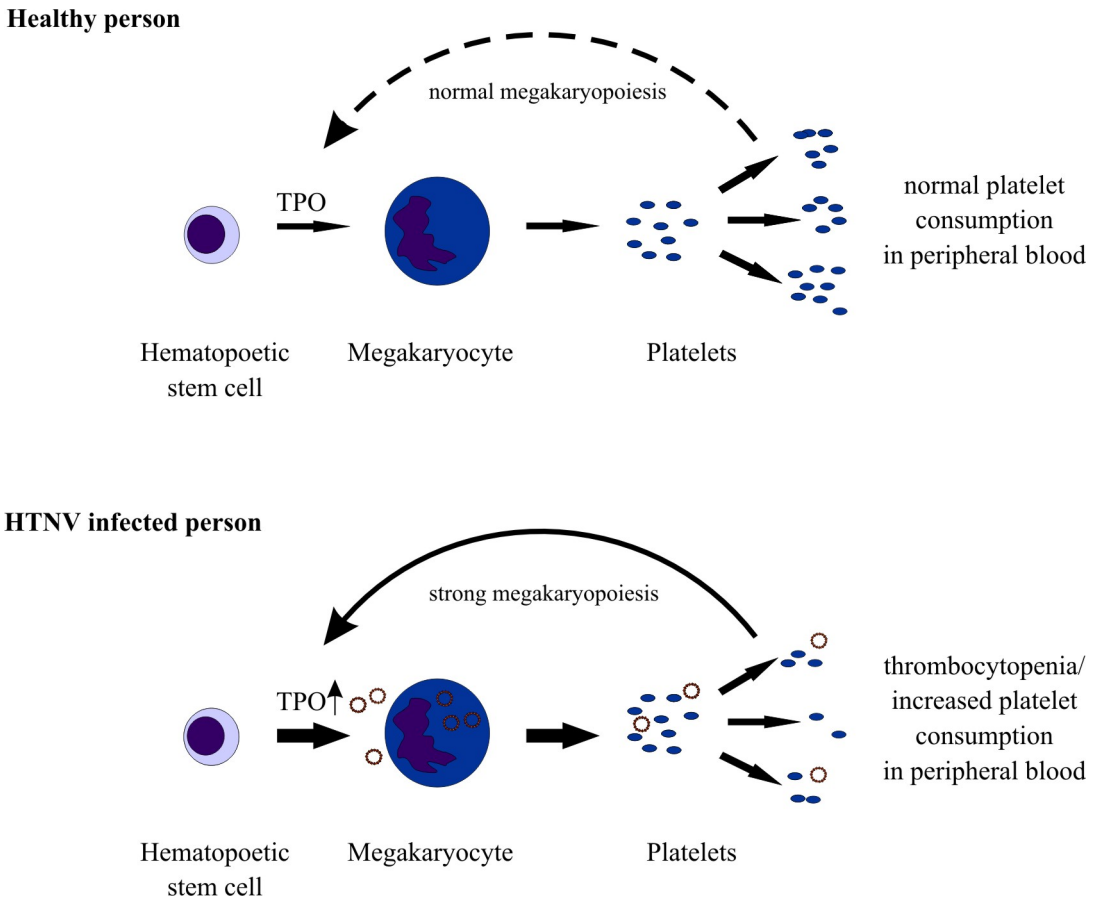

Figure 37: Regulation of megakaryopoiesis in a healthy and a HTNV-infected person.

In a healthy person TPO expression is constant and regulates formation of megakaryocytes and platelets. An autoregulatory loop controls this process. In a hantavirus-infected person numbers of platelets are decreased. Via signals of the autoregulatory loop TPO serum levels become slightly increased. Thus megakaryocyte formation is enhanced to ensure physiologic platelet count. Thereby more virus producing cells are available, increasing overall amount of viral particles.

Secondly, hantaviruses can interact with human platelets without productively infecting them. Expression of important adhesion molecules is reduced on platelets exposed to HTNV, indicating reduced platelet function.

4.5 Consequences for the natural host

Although not analysed yet, it is possible that hantaviruses infect megakaryocytes in the bone marrow of their natural reservoir hosts, offering a strategy to establish persistent infection. In contrast to humans, the antiviral CTL response is inhibited by regulatory T-cells in the natural reservoir hosts (Easterbrook *et al.*, 2007; Schountz *et al.*, 2007). This could prevent virus elimination resulting in persistent and lifelong infection of cells of the megakaryocytic lineage. Intriguingly, megakaryocytes/proplatelets accumulate in the lung of humans and rodents (Bozza *et al.*, 2009). Thus infected cells from the bone marrow could transport the virus to the lung. In accordance with this view, hantavirus RNA has been detected in the lungs of reservoir hosts during persistent infection (Botten *et al.*, 2003; Botten *et al.*, 2002; Lee *et al.*, 1986). Increasing platelet demand after wounding leads to increased megakaryocyte formation in the bone marrow (Deutsch & Tomer, 2006) enlarging the pool of cells, which produce hantaviral virions. Such a mechanism could increase the virus load in the blood of wounded animals and thus the likelihood for virus transmission. In line with this notion aggressive behaviour and wounding correlate with virus transmission (Easterbrook *et al.*, 2007; Escutenaire *et al.*, 2002; Glass *et al.*, 1988). Future studies have to clarify whether primary megakaryocytes are infected in the natural reservoir host and contribute to persistent infection.

4.6 Lymphocytes and monocytes

A key finding in hantavirus infection is a strong antiviral immune response (Maes *et al.*, 2004; Schonrich *et al.*, 2008). Analysis of lymphocytes after infection with HTNV *in vitro* showed a strong upregulation of activation marker CD69 on respective cells. CD69 is involved in lymphocyte proliferation and functions as a signal-transmitting receptor. This indicates that HTNV triggers strong lymphocyte activation making them potent effector cells. Furthermore, we could show that HTNV prevents apoptosis in monocytes isolated from healthy human donors as compared to mock-infected monocytes (Schonrich *et al.*, 2008). Most importantly, these HTNV-infected monocytes developed into DC-like cells. Similar results were found by other researchers (Markotic *et al.*, 2007). Monocytes can differentiate into DC like cells after stimulation with IFN-α and GM-CSF (Mohty *et al.*, 2003). This suggests that expression of these cytokines is

induced in HTNV-infected monocytes, indicating that HTNV triggers an innate immune response in human monocytes. It is possible that these DC-like cells also contribute to viral dissemination as suggested for immature dendritic cells (Raftery *et al.*, 2002). These HTNV-infected DC-like cells could play a role in inducing a dysregulated antiviral immune-response. Moreover, the unusual strong and longlasting T-cell memory described previously for patients infected many years ago with PUUV (Van Epps *et al.*, 2002), could also be in part due to development of DC-like antigen-presenting cells. Finally, hantavirus-infected immune cells could activate uninfected bystander through secretion of cytokines. In line with this notion lies the observation that in infected patient's serum levels of TNF-α (tumor necrosis factor-α) , IFN-γ, IL-6, IL-2 and IL-10 were elevated (Linderholm *et al.*, 1996; McCaughey & Hart, 2000; Zaki *et al.*, 1995). Altogether, these results support the concept of immunopathogenesis during hantavirus infection.

4.7 Hantavirus-associated immunopathogenesis

In conclusion, the results of this thesis strongly support the hypothesis of virus-induced immunopathogenesis in humans (figure 38). In the human host hantaviral virions are taken up via aerosols. Thus, the lung is the first site of infection. Several cell types that are found in the lung are susceptible to hantavirus infection: endothelial cells, dendritic cells, monocytes and megakaryocytes, These cells could serve as Trojan horses that disseminate the virus in the organism. As in humans no inhibitory T-reg cells are induced by hantaviruses, activation of the immune system is not suppressed. Subsequently, a strong cytotoxic antiviral immune response develops, characterized by release of cytokines, differentiation of infected monocytes into DC-like cells, dendritic cell maturation, activation of lymphocytes and CTLs. The latter could attack infected cells thereby destroying the endothelial cells and megakaryocytes, which leads to thrombocytopenia. Furthermore, the coagulation ability of platelets may be diminished. As not enough functional platelets are available the lesions in the damaged endothelial barrier cannot be sealed, and haemorrhages develop.

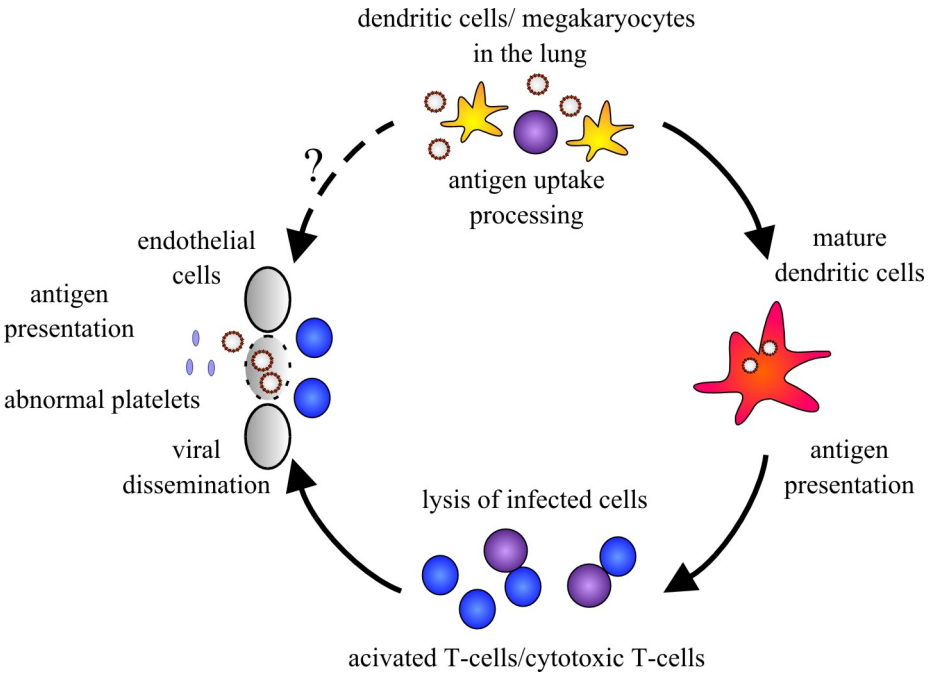

Figure 38: Expanded working hypothesis of antiviral immunopathogenesis in humans.

Next to the dendritic cells in the lung megakaryocytic cells can be infected. Thus these cells are becoming targets for CTLs. Once megakaryocytes are attracted by cytotoxic T-cells no more platelets can be formed leading to thrombocytopenia. As less platelets are available, lesions in the endothelial barrier cannot be fixed anymore, leading to serious haemorrhages.

4.8 Outlook

For the first time we showed that pathogenic, but not apathogenic hantaviruses interact with cells of the haemostatic system. We infected human megakaryocytes with HTNV making those cells targets for the immune system and we demonstrated that platelets and pathogenic hantaviruses interact, resulting in decreased expression of surface proteins essential for platelet function. Moreover we showed strong activation of human lymphocytes and differentiation of monocytes into DC-like cells after infection strongly supporting the concept of hantavirus-associated immunopathogenesis.

Further research in this field should concentrate on studies to establish a suitable animal model to clarify whether the concept of hantavirus-induced immunopathogenesis holds true or whether not yet defined direct viral effects are decisive for the development of human disease. Along this line humanized mice that are not only equipped with a human immune system but also generate human platelets from human megakaryocytes, could be infected with pathogenic hantaviruses. In this way one could test whether the antiviral immune response is indeed responsible for hantavirus-induced

acute thrombocytopenia *in vivo*. With this approach, it could also be clarified whether pathogenic hantaviruses affect the clotting ability of platelets. Another interesting question will be to investigate the differences between HTNV and other pathogenic hantaviruses in megakaryocytic cell lines and humanized mice. Finally, we may not forget to study the role of megakaryocytes and platelets in the natural host, where the immune response against hantaviruses is downregulated by regulatory T-cells. This suggests that infection of megakaryocytes could contribute to lifelong persistence in the natural host.

All these questions show the urgent need for a suitable animal disease model of hantavirus infection. This would strongly facilitate basic research and enable us to understand the precise mechanisms underlying hantavirus-associated human disease. Based on this knowledge it will be possible to design effective therapies that help patients suffering from NE, HFRS or HCPS.

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und nur unter Verwendung der genannten Hilfen und Hilfsmittel verfasst habe.

Nina Lütteke

List of publications

Hantavirus-induced immunity in rodent reservoirs and humans;
Schönrich G, Rang A, Lütteke N, Raftery MJ, Charbonnel N, Ulrich RG;
Immunol Rev, 2008 Oct, 225:136-89

Switch to high-level virus replication and HLA class I upregulation in differentiating megakaryocytic cells after infection with pathogenic hantavirus
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